MATRIX ATTACHMENT REGIONS AND TRANSGENE EXPRESSION

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INTRODUCTION

Many of the questions we have about how biological systems work are ultimately questions about the regulation of gene expression. For this reason, the control of transcription is fundamental and has received well-deserved attention. From a simplistic point of view, transcription can be though of as being regulated at two levels. The first level (coarse control) involves access of RNA polymerase and transacting factors to the specific DNA sequences with which they interact. Access is a function of chromatin structure. In chromatin fibers of both condensed metaphase chromosomes and interphase chromatin, DNA is highly compacted and essentially inaccessible to RNA polymerase and trans-acting factors. In order to make the DNA accessible, chromatin fibers must in some way take on a more open, less compact structure. Once an open (transcriptionally poised) chromatin structure is obtained, further regulation of transcription involving availability and interactions of transacting factors (fine control), come into play. For reviews see Paranjape et al. (1994) and Reeves (1984).

One of the hallmarks of transcriptionally poised chromatin is heightened general sensitivity to the endonuclease DNase I (Weintraub and Groudine, 1976). 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

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transcriptionally active chromatin (as assayed by DNase I sensitivity) correspond to structural domains in chromatin that have been described as "loop domains" (Bode and Maass, 1988; Bonifer et al., 1991; Farache et al., 1990; Gasser and Laemmle, 1986; Levy-Wilson and Fortier, 1989; Mirkovitch et al., 1984; Phi-van and Strätling, 1988).

Over the last several years abundant evidence has appeared in support of the loop domain model of chromatin structure (reviewed in Laemmli et al., 1992). According to this model, chromatin fibers are organized into a series of loops attached at their bases to a proteinaceous network called the nuclear matrix. The loops range in size form about 5 kb to more than 100 kb and attachment is through a specific interaction between proteins of the nuclear matrix and DNA sequences (approximately 1 kb) called Matrix Attachment Regions (MARs[1]) (see Figure 1). The loop domains have been hypothesized to form topologically isolated units of transcription and replication (Amati and Gasser, 1988; Bonifer et al., 1991; Goldman, 1988; Jack and Eggart, 1992).

Some early experiments with transfected animal cells in culture indicated that MAR sequences can overcome the problems of low and variable expression of introduced genes that have traditionally been attributed to "position effects" - i.e., to the site of insertion of transgenes into host chromatin in transfected cell lines. In these experiments, introduced genes were flanked with MAR sequences with the result that the average level of gene expression increased approximately tenfold and the formerly variable expression became copy-number dependent (Bonifer et al., 1990; Phi-Van et al., 1990; Stief et al., 1989). An early model to explain these results is depicted in Figure 1. According to this model, an introduced gene can become incorporated into the host chromatin in either an open, transcriptionally poised domain (Figure 1A, left) or a highly coiled, transcriptionally inactive domain (Figure 1A, right). An introduced gene without flanking MARs takes on the chromatin structure of the domain into which it becomes incorporated. Because most domains in multicellular eukaryotes would be expected to be inactive domains, most introduced genes would have a transcriptionally inactive chromatin structure. Only genes that become incorporated into transcriptionally active domains would be expected to have a high potential for transcription. Conversely, when an introduced gene is flanked by cloned MARs, it can form its own independent loop domain insulated from the structure of the domain into which it becomes incorporated (Figure 1B). There is no *à priori* reason to believe that the artificial domains created by the cloned MARs should be

[1]The nuclear matrix and MARs have also been called the nuclear scaffold and SARs. Although there is a formal distinction between the two sets of terms, based on method of preparation, the biological concept is the same. We will use the terms Matrix and MARs in this work.

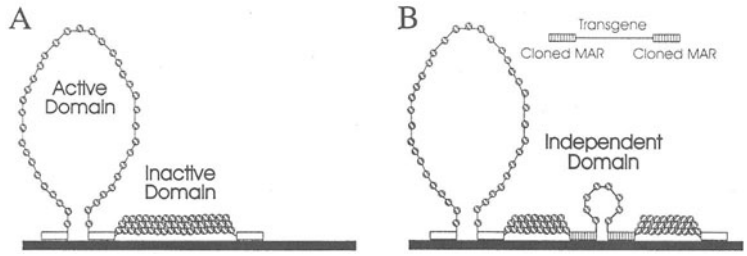

Figure 1. Models depicting the organization of chromatin into active and inactive loop domains and the formation of independent transgenic loop domains. **A.** MAR sequences (open boxes) interact with nuclear matrix fiber (filled bar) to form two loop domains. The active domain is depicted as a 11-nm 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.

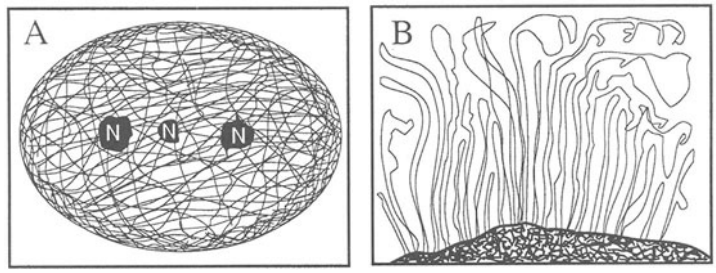

Figure 2. Nuclear matrix and halo structures. **A.** Drawing based on an electron micrograph (Capo et al., 1982) of a nucleus from which the nuclear membrane has been removed by detergents and chromatin removed by high salt extraction leaving the fibers of the nuclear matrix. N=nucleoli. **B.** Drawing 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).

transcriptionally poised domains as shown in the figure, but a tenfold increase in average gene expression and copy-number dependence would indicate that they are.

The prospect of overcoming "position effect" problems has created widespread interest in the use of MARs in transgenic organisms. Work in this field is still in the early stages but it is clear that the effects of MARs in transgenic organisms cannot be accounted for solely by the model shown in Figure 1. The early observations of MAR-mediated increases in gene expression have in general been corroborated. Sometimes the increases have been much greater than tenfold, but in a few instances they have been negligible or nonexistent. MAR-mediated reduction in variability of transgene expression has not been consistently demonstrated. Because several experiments have demonstrated lack of a simple correlation between copy number and gene expression, the idea that MARs increase gene expression by overcoming "position effects" has been questioned. In the following pages we will discuss some of the properties of MARs and examine the evidence concerning their effects on the expression of transgenes. We will also discuss some of the models that have been put forth to explain the biological activity of MARs and how our interpretation of data may be complicated by the effects of homology-dependent gene silencing. We will use data from all biological systems in our attempts to describe the current state of knowledge in the field, but we will place emphasis in examining the data from plant systems.

THE NUCLEAR MATRIX AND MATRIX ATTACHMENT REGIONS

After years of controversy, the existence of a nuclear matrix seems to be well established (Jack and Eggert, 1992). Figure 2A is a drawing representing a transmission electron micrograph of a portion of a nucleus (including nucleoli) from which chromatin has been removed leaving the residual, proteinaceous fibers of the nuclear matrix. Figure 2B is another drawing representing a portion of a chromosome scaffold with loops of DNA emanating from it. In the original micrograph upon which this figure is based, histones have been extracted from a metaphase chromosome, thus removing the soiling restraints and allowing the DNA to spill out and form a "cloud" or "halo" around the chromosome scaffold. The DNA sequences at the point of attachment of the loops to the scaffold or matrix fibers are the MARs. There is a great deal of evidence indicating that the MARs are not random-sequence DNAs, but rather that the MAR-nuclear matrix interaction is specific. As will be discussed below, the sequences required to bestow matrix-binding activity are imprecisely known. MARs in general are very AT-rich (typically>70%), but high AT-content alone does not insure matrix binding. A number of MAR consensus sequences have been proposed, but the relevance of the consensus sequences is questionable because they all are very AT-rich and might be expected to occur frequently by chance in such high AT-content DNA.

MARs can be identified by two assays. The endogenous assay identifies DNA sequences as MARs by their co-isolation with purified nuclear matrices. The exogenous assay identifies MARs by their ability to bind isolated nuclear matrices in vitro. The isolation of the nuclear matrix and the endogenous and exogenous MAR assays are outlined in Figure 3. The procedure begins with isolated nuclei. Histones and other chromatin proteins are removed form isolated nuclei by a variety of treatments. We use the procedure involving extraction with lithium diiodosalicylate (LIS) (Hall et al., 1991; Hall and Spiker, 1994; Mirkovitch et al., 1984). Removal of histones results in formation of nuclear halos (compare to Figure 2B) The DNA in the loops is then solubilized with endonucleases (usually restriction enzymes). The solubilized DNA can then be separated form the insoluble nuclear matrices by centrifugation. In the endogenous assay the solubilized DNA (DNA in the loops) and the DNA associated with the insoluble matrices (MARs by operational definition) can be assayed in Southern blots probed with any DNA sequence present originally in the nuclei. The exogenous assay is not so limited. In this assay end-labeled DNA from any source is incubated with the isolated nuclear matrices along with competitor DNA. MAR sequences will bind to the insoluble nuclear matrix and thus be found in the pellet after centrifugation. Non-MAR sequences remain in the supernatant fraction.

Figure 4 shows examples of "endogenous" assays (4A) and "exogenous" assays (4C) with tobacco (NT1 cells). In Figure 4B is a map of a portion of a genomic clone harboring a tobacco root-specific gene that has been called RB7 (Conkling et al., 1990). The coding portion of the gene is represented by a 1.8 kb HindIII fragment marked with an "a" and with an arrow to show the direction of transcription. Several kb downstream from the coding region is an XbaI fragment, marked with a "b," in a region that contains a MAR. In the endogenous assay (4A), DNA form the matrix (pellet;P) and supernatant (S) fractions are isolated and equal quantities are separated on agarose gels and blotted as outlined on the right side of Figure 3. A lane of restriction-enzyme digested total DNA (T) is included for comparison. The blots are probed with the "a" fragment, representing the coding region (left three lanes) or the "b" fragment, representing the region of the MAR DNA (right three lanes). Note that when the coding region ("a") is used as a probe, HindIII is used in the nuclear matrix preparation and to digest the total DNA used for comparison. In this case the DNA hybridizing to the probe is essentially all in the supernatant lane, indicating that the DNA in this region does not contain a MAR. When the "b" fragment is used as a probe, EcoRI is used in the nuclear matrix preparation and to digest the total DNA. Here, essentially all the DNA hybridizing to the probe is found in the pellet lane, indicating that fragments containing the "b" DNA contain MARs. Note that a prominent 4.3 kb hybridizing fragment is found in the pellet lane. This may indicate that one of the EcoRI sites is partially protected by interaction with the nuclear matrix.

In Figure 4C, two "exogenous" assays are shown. Purified nuclear matrices are made and incubated with end-labeled fragments as shown on the left side of Figure 3. Competitor DNA in both assays is tobacco genomic DNA. After incubation, DNA bound to the insoluble nuclear matrix (MARs by operational definition) is separated form non-bound DNA by centrifugation. Both DNA populations, along with a lane (total, T) of labeled fragments that were not incubated with the nuclear matrix preparations, are subjected to agarose gel electrophoresis and analyzed directly by autoradiography. In the left three lanes a plasmid containing an insert of the coding region in a HindIII site, was digested with HindIII and end-labeled. The vector (V) and the insert (H-H) are both found in the supernatant. Thus neither contains MAR DNA. In the right three lanes a plasmid containing an insert at the HindIII/SalI sites is cut with HindIII, SalI and XbaI. The fragments are end-labeled, incubated with the nuclear matrix and analyzed in the same way. Again the vector fragment (V) partitions with the supernatant as it does not contain a MAR. The XbaI-SalI (X-S) fragment also does not contain a MAR. The XbaI-XbaI (X-X) fragment partitions almost entirely with the pellet.

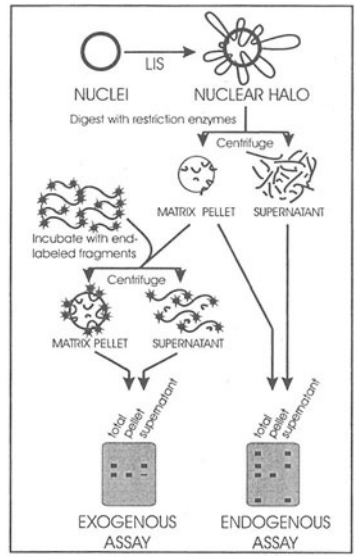

Figure 3. Isolation of the nuclear matrix and characterization of MARs by exogenous and endogenous assays. Chromatin proteins are extracted form isolated nuclei (we use lithium diiodosalicylate, LIS) thus removing coiling restraints and allowing DNA to spill out and form a nuclear halo (compare to Fig. 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 and Figure 4 for examples.

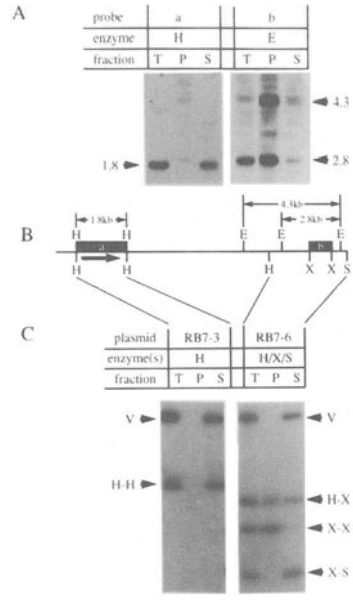

Figure 4. Examples of the endogenous and exogenous nuclear matrix binding assays using a genomic clone from the tobacco root-specific RB7 gene (see text). In panel B is a map of the RB7 gene indicating probes used for the endogenous assay (panel A) and the exogenous assay (panel C). The solid arrow in panel B shows the position and orientation of the RB7 coding region. On the top part of the map, restriction sites and fragment sizes are shown as they pertain to the endogenous assay. *Endogenous assay:* Probe= DNA fragments used to probe Southern blots, as shown in panel B; enzyme= restriction endonucleases used to digest nuclear halos; H= HindIII; E=EcoRI; fraction= the fraction of DNA run on the gel for the Southern blot; T= total purified tobacco (NT1) genomic DNA; P= pellet or matrix-bound fragment; S= supernatant or released fraction. Cutting is inefficient in the middle EcoRI site, resulting in a prominent 4.3 kb fragment. This site may be partially protected due to matrix association. *Exogenous assay:* Plasmid- subclones used to make end-labeled probes (RB7-3 contains a 1.8 kb HindIII/HindIII fragment and RB7-6 contains a 3.4 kb HindIII/SalI fragment); enzymes= restriction enzymes used to make the end-labeled probes; fraction= the fraction of DNA run on the gels; T= total input end-labeled fragment; P= pellet or matrix associated fraction; S- supernatant or unbound fraction. For these assays, equal proportions of each of the fractions are loaded onto a gel and detected by direct autoradiography after electrophoresis. Arrows indicate the bands of interest. V= vector; other fragments are identified by the restriction sites at their ends. (Reproduced form Hall and Spiker, 1994).

Thus, this fragment contains a MAR, and the MAR is strong. DNA containing this strong tobacco MAR was used in our experiments (described below) to test the effects of MARs in transgene expression in plant cells. The HindIII-XbaI (H-X) fragment also contains a MAR. This MAR is much weaker, however, as it partitions about equally between the pellet and supernatant. The yeast MAR that we used in our initial investigations of the effects of MARs on transgene expression in plant cells (Allen et al., 1993) was even weaker than the H-X fragment. There appears to be a correlation between matrix binding activity and effect on gene expression, as will be outlined below.

One might ask if the difference in apparent affinities of the weak yeast MAR and the strong tobacco MAR for the tobacco nuclear matrix might be explained by species specificity. This does not appear to be the case. We have characterized a large number of tobacco MARs (many of which were isolated by cloning DNA bound to isolate nuclear matrices) and found apparent affinities ranging from that of the yeast MAR to that of the strong tobacco MAR (Susan Michalowski, unpublished data).

What then determines if a DNA fragment will bind specifically to isolated nuclear matrices? A number of sequences have been found to be part of MARs. Among the first to be noted were the "A-box" (consensus AATAAAT/CAAA) and the "T-box" (consensus TTA/TTA/TTTA/TTT) (Gasser and Laemmli, 1986). Gasser and Laemmli also noted frequent occurrence of matches to the six bp core of the *Drosophila* topoisomerase II consensus sequence (A/TAT/CATT). Bode and co-workers (Bode et al., 1992) have stressed the importance of DNA sequences that can serve as nucleation sites for DNA unwinding including the sequence AATATATTT. Boulikas (1993) has suggested other consensus sequences. It is probable, however, that searches for consensus sequences are unlikely to tell us what determines if a DNA sequence can bind to the nuclear matrix. Part of the difficulty is that MARs are large. They are usually in the range of 1000 base pairs, although regions of direct contact with the matrix may be smaller (Gasser and Laemmli, 1986B). MAR affinity for the nuclear matrix is somewhat size dependent (around 300 bp is the lower limit for the exogenous MAR assay; see Gasser et al., 1989). Large MARs can often be cut into smaller pieces with each of the smaller pieces retaining matrix binding capacity, albeit often with lower apparent affinity (Hall et al., 1991). Thus is appears as though overall structural features of AT-rich DNA, rather than sequence per se determine matrix affinity of MARs. DNA sequences containing tracts of A/T have a number of unusual properties including a narrow minor groove, reduced capacity to form nucleosomes, tendency of phased runs to form bent DNA structures (Nelson et al., 1987) and tendency toward singe-strandedness (Bode et al., 1992). The idea that overall structure, rather than sequence per se, dictates matrix binding is supported by a correlation between apparent binding strength and numbers of runs of twenty base pairs of at least 90% AT nucleotides (S. Michalowski, unpublished data). We cannot as yet specify structural features of DNA that confer matrix binding. Although high AT-content appears to be involved, it is clear that high AT-content alone is not enough. Several At-rich fragments have been shown to lack matrix-binding capacity (Amati and Gasser, 1988; Bode et al., 1992; Gasser and Laemmli, 1986; Jarmon and Higgs, 1988).

It is obvious that the nuclear matrix is a complex structure containing many different types of proteins (Beven et al., 1991; Capco et al., 1982; Grabher et al., 1992; Hall et al., 1991; Ivanchenko and Avramova, 1992; Ivanchenko et al., 1993; Krachmarov et al., 1991: McNulty and Saunders, 1992). Several approaches have been used to

investigate the proteins involve in binding MAR sequences. For example, screening an expression library for clones producing MAR-binding proteins resulted in the isolation of a cDNA for the SATB1 protein (Dickinson et al., 1992). Cross-linking procedures have been used (Dworetzky et al., 1992; Ferraro et al., 1992) as has Southwestern blotting (Romig et al., 1992; Tsutsui et al., 1993; von Kries et al., 1991). If there is a single protein that is responsible for the bulk of the MAR binding, such a protein has not as yet been identified. At present several seemingly unrelated proteins have been shown to have such an activity in vitro. In addition to the proteins identified by the methods mentioned above, other proteins including H1 histones, HMG proteins and topoisomerase II have been shown to interact with MAR sequences in vitro (Adachi et al., 1989; Dickinson et al., 1992; Ivanchenko and Avramova, 1992; Izaurralde et al., 1989; von Kries et al., 1991; Zhao et al., 1993) Of these, H1 histones would not be expected to be bonafide nuclear matrix proteins, although they often appear in nuclear matrix preparations (Capco et al., 1992). HMG proteins also would not be expected to be a part of the nuclear matrix. There is one report indicating that HMG proteins are a part of the nuclear matrix (Ivanchenko and Avramova, 1992), but we find no evidence for NMG proteins in plant nuclear matrix preparations by a Western blotting approach (T. Phelan, unpublished data). Earnshaw and Heck (Earnshaw and Heck, 1995; Earnshaw and Heck, 1988) have demonstrated that topoisomerase II is a major protein associated with metaphase chromosome scaffolds in chicken cells grown in culture. This is an intriguing observation as it evokes an image of topoisomerase II residing at the base of a chromosomal loop domain and directly controlling the topology of the DNA in that domain. The generality of such a role for topoisomerase II must be questioned, however, because this protein does not appear to be a part of the chromosome scaffold in quiescent tissues (Anderson and Roberge, 1992).

EFFECT OF MARS ON TRANSGENE EXPRESSION IN ANIMAL SYSTEMS

Several papers have been published concerning the effect of MAR sequences on expression of stably integrated genes in cells and organisms. In general, the results of these experiments have been interpreted to demonstrate that MARs increase levels of expression and decrease variability of expression. In some cases "position-independent" and "copy number-dependent" expression has been claimed, although it is not always clear precisely what is meant by these terms. Strictly speaking, position independence should mean that all single copy transformants (discounting deletions and rearrangements) would transcribe the transgene at essentially the same rate regardless of location in the genome. In the absence of complicating phenomena, additional copies of the transgene should

also be transcribed at the same rate, resulting in a simple linear relationship between copy number and total expression. In practice, however, variability in expression of transgenes flanked by MARs is still considerable. In fact, in absolute terms, variability in expression of transgenes flanked by MARs is usually greater than controls--not surprising in view of the much higher overall levels of expression of MAR-bounded transgenes. 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 (standard deviation divided by the man) as a measure of variability does the decrease in variation become apparent (Allen et al., 1993; Mlynárová et al., 1995; Mlynárová et al., 1994; Phi-Van et al., 1990).

In the first paper specifically designed to test the effects of MARs (Stief et al., 1989) a reporter gene, CAT (Chloramphenicol acetyl transferase) with promoter and enhancer was flanked by chicken lysozyme MARs and used to transform chicken promacrophage cells by transfection. In these experiments, the overall CAT expression form the MAR transformants was about 10-fold greater than the non-MAR controls. Variability of expression in low copy number MAR transformants was slightly less than 10-fold. Non-MAR transformants varied slightly more than 10-fold. Thus, although variability in expression was reduced somewhat, convincing evidence for position-independent gene activity is lacking according to the criteria mentioned above. In the MAR transformants, higher copy number (up to nearly 30 copies per genome) resulted in generally higher levels of expression, although the correspondence between copy number and expression levels was not spectacularly better than in controls. In parallel experiments, similar constructs lacking the enhancer element were used. Again an approximate 10-fold increase in CAT expression was realized in the MAR transformants, but there was no evidence for position independent or coy number dependent expression. Thus, in this work there is some support for MAR-mediated reduction in variability, but the most obvious and striking effect of the MARs is the 10-fold increase in average gene expression. It should be noted that in this paper most of the work was done with measurement of CAT activity, but it was demonstrated that CAT activity correlated with steady-state levels of CAT messenger RNA. These workers also showed that the effect of MARs is dependent upon stable integration into the host genome. In transient expression assays, the MAR sequences had either no effect or a slightly inhibitory effect. That MARs must be stably integrated into the host genome in order to have their effects has been consistently demonstrated (Allen et al., 1993; Klehr et al., 1991).

In a similar set of experiments, Phi-van and co-workers (Phi-Van et al., 1990) used the same reporter gene and the same MAR to transfect rat fibroblasts. Very few low copy number transformants were produced. Thus, the claims for dampening of position effects were based on copy number dependency of gene expression. The evidence here is somewhat more convincing than that of Stief et al.

(1989), and certainly the claim of dampening of position effects is well supported. Nevertheless, the most obvious effect of MARs in these experiments is the approximate 10-fold increase in transgene expression.

In another early paper, Klehr and co-workers (Klehr et al., 1991) used a variety of MARs (including a MAR of plant origin) and found a 20 to 30-fold enhancement of transcription in transfected mouse L cells. No claims for reduction in variability of expression were made in this work.

Determining MAR effects in transgenic animals has been less straightforward than it has been in cells in culture. Bonifer and co-workers (Bonifer et al., 1990) introduced a 21.5 kb fragment carrying the entire chicken lysozyme gene locus into the germ line of mice by injection into fertilized oocytes. In the resulting transgenic animals, a good correspondence between copy number (up to 70) and gene expression was demonstrated. Evaluation of the data is complicated by the fact that only 7 mice were analyzed, and that the DNA introduced contained may sequences in addition to the MARs. Further studies by these same investigators (Bonifer et al., 1994) in which selective regions of the 21.5 kb fragment were deleted, indicated that an interplay between the MAR elements and a locus control region (LCR) affects the level and tissue specificity of transgene expression. LCRs are regions with high densities of DNase I hypersensitive sites that are thought to be involved in determination of chromatin structure in native, developmentally regulated chromatin domains.

McKnight and co-workers (McKnight et al., 1992) used a similar germ-line injection procedure to investigate the effect of the chicken lysozyme MAR on the expression of the mouse whey acidic protein in transgenic mice. In this study, no evidence for MAR-mediated higher overall expression or copy-number dependency were found. Position-independent regulation was claimed, however, based on the effect of MARs on tissue specificity and hormonal regulation.

Based on studies such as those just mentioned, the generalization has arisen that MARs can mediate increased levels of overall gene expression, reduce transformant to transformant variability in transcription, dampen position effects and allow copy number-dependent expression. We have recently published work (Allen et al., 1993) that confirms MAR-mediated increases in gene expression in transformed plant cells. Our data, however, conflicted with published data from animal systems in that copy-number-dependent gene expression was not observed. In fact, high copy number appeared to inhibit transgene expression. We rationalized The differences between our data and the data from animal systems by postulating that homology dependent gene silencing is more prevalent in plant systems than in animal systems (see later).

However, two recently published papers on animal systems have presented data, which appear to correspond more closely to our work with transformed tobacco cells than to previous work with animal cells (Kalos and Fournier, 1995; Poljak et al., 1994). Poljak and co-workers

used the CAT reporter gene driven by the SV40 promoter and enhancer. This construct, either alone or flanked by MARs from *Drosophila* histone or heat shock gene, was transfected into HeLa cells or mouse L cells. The MAR sequences did not reduce variability in expression, nor did they result in copy number-dependent expression. In fact, in accordance with our data from tobacco cells, CAT expression per DNA copy was dramatically reduced at higher copy numbers. Overall, MAR-mediated stimulation of transgene activity was about 40-fold. The authors conclude that MARs stimulate transgene expression but do not confer position-independent expression, and invoke a model in which MARs are considered to increase expression by providing entry points, or nucleation sites, for HMGs or related proteins to replace H1 histones. However, they do not explain how their data differ from previously published data on animal cells in culture.

Kalos and Fournier (1995) used both human and rat hepatoma cells for their experiments on human apolipoprotein B (*apoB*) 5' and 3' MARs. The reporter gene was ß-galactosidase driven by the *apoB* promoter. For analysis, these workers divided the transformed cell lines into 1-2 copy lines and multicopy lines. In the 1-2 copy lines, the cells transformed with MAR-containing constructs showed consistently higher expression than control lines (approximately 200-fold). The authors attributed the MAR effect to position-independent transgene expression. In stark contrast to the results of Stief et al., (1989) and Phi-Van et al., (1990), Kalos and Fournier did not observe copy number dependent expression in their experiments. In fact, in their multicopy lines, transgene expression was strongly repressed. The difference between these data and the early data on the effects of MARs on transgene expression remains unexplained.

MAR EFFECTS IN PLANT SYSTEMS

In plant systems, most of the reported experiments have used *Agrobacterium* and T-DNA vectors for transformation. An initial report by Breyne et al. (Breyne et al., 1992) described a slightly negative effect of MARs on reporter gene expression, but there are now several reports of moderate increases, averaging a few fold in magnitude (Table 1) (Mlynárová et al., 1995; Mlynárová et al., 1994; Schöffl et al., 1993; van der Geest et al., 1994). In contrast, we have shown that a yeast MAR contained in the *ARS-1* element can increase average expression of a *35S::GUS* reporter gene more than 20 fold (per transgene copy) in tobacco suspension culture cells stably transformed by microprojectile bombardment (Allen et al., 1993). Thus, it is clear that large MAR effects can be obtained in plant cells under appropriate circumstances, and that MARs from other organisms can work in plants.

Additional experiments with a MAR derived from the tobacco genome (Allen et al., 1995) have produced even larger effects, with the

increase in expression per transgene copy averaging over 130 fold. This particular MAR was isolated from a genomic clone of the *RB7* gene kindly provided by our colleague, Mark Conkling (Conkling et al., 1990) and shown to bind very strongly to tobacco matrix preparations (Hall et al., 1991). It is tempting to speculate that its greater effect on expression in vivo is related to its stronger matrix binding activity in vitro, but further investigation well be required before this issue can be properly resolved.

The lower magnitude of the MAR effects obtained with T-DNA may be a function of the close linkage between the selectable marker and the reporter genes in these vectors. We assume that most, although probably not all, cases of inactivation involve both the selectable marker and the reporter gene, and thus will be eliminated by drug selection. Since many such events are thus excluded from the analysis, any MAR-mediated reduction in their frequency will be underestimated. Viewed from this perspective, it is remarkable that any MAR effect can be observed in such experiments.

Table 1. MAR Effects in Transgenic 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*	Bombard-ment	12 fold increase	small decrease	Allen et al. (1993)
Tobacco plants	Chicken	*Lhca3-GUS*	T-DNA	4 fold decrease	3 fold	Mlynárová et al. (1994)
	Chicken	*Lhca3-GUS*[1]	T-DNA	3 fold decrease	7 fold	
Tobacco plants	Bean	Phaseolin-*GUS*	T-DNA	3 fold decrease	2 fold	van der Geest et al. (1994)
Tobacco plants	Chicken	*Enh35S-GUS*	T-DNA	2 fold decrease	2 fold	Mlynárová et al. (1995)
	Chicken	*Enh35S GUS*[1]	T-DNA	2 fold decrease	7 fold	
Tobacco cells	Tobacco	*35S-GUS*	Bombard-ment	60 fold increase	no effect	Allen et al. (1995)

1. In these constructs, the selectable marker and the reporter gene were between the MARs in a single domain.

In contrast, the microprojectile bombardment experiments employed a co-transformation procedure in which the reporter gene and selectable marker (the *NptII* gene) were delivered on separate plasmids. Our data on NptII protein levels indicate that this gene is unaffected by the MARs on the GUS plasmid. Thus, we have been able to achieve an operational separation between selectable marker and reporter genes in this system, in spite of the known tendency for co-transformed plasmids to integrate at the same genetic locus (Christou et al., 1989; McCabe et al., 1988; Peng et al., 1995; Registar et al., 1994).

Wherever tested, these MAR effects require that the transgene DNA be integrated into the host plant genome. Only small effects are seen in transient expression assays, consistent with the notion that MARs work by altering chromatin structure rather than as classical enhancers.

DO MARS REDUCE POSITION EFFECTS?

It is clear from work cited above that MARs can increase transgene expression in both plants and animals, sometimes by a very large factor. Although it does not necessarily follow from this result that MARs should also reduce position effect variation, there are several reports suggesting that in fact they do. In most instances, these data come from experiments with stably transformed cell lines or transgenic plants. Without MARs, gene expression varies from line to line in a largely random fashion, showing little or no dependence on gene copy number. However, when MARs are placed at the ends of otherwise identical constructs there is an increase in the copy number dependence and/or a decrease in the apparently random variation normally attributed to position effect.

A Elements and Copy Number Dependence

Interestingly, most instance in which MARs have been reported to reduce position effect variation involve the so-called "A element" from the chicken lysozyme gene. This element is the 5' MAR in a chromosomal domain studied by Bonifer et al., (1990). These authors tested a 21.5 Kb fragment from the chicken genome containing the entire lysozyme gene domain, including all known regulatory elements and both 5' and 3' MARs. When the entire domain was introduced into transgenic mice, it showed developmentally appropriate expression that was proportional to transgene copy number, indicating that it functions as a position independent regulatory unit.

The 5' MAR from this domain was also tested in chimaeric gene constructs in which it was placed both 5' and 3' to a reporter gene (Phi-Van et al., 1990; Stief et al., 1989). In both cases, overall expression in the transformant population was increased significantly, and the expression of individual cell lines varied in rough proportion

to transgene copy number. These results were interpreted as indicating that MARs could insulate genes from chromosomal position effects.

In plant systems, the best evidence for reduction in position effect variation also comes from experiments with the chicken lysozyme A element. Nap and his colleagues have studied a large number of transgenic tobacco plants prepared with reporter gene constructs with or without flanking A elements (Mlynárová et al., 1995; Mlynárová et al., 1994). Moderate increases in average GUS expression (roughly 2 to 5 fold) were obtained, coupled with a dramatic reduction in the number of plants in which activity was low or undetectable. The reduction in the number of low expressors is consistent with the hypothesis that MARs substantially reduce the impact of gene silencing effects, and that such effects contribute a significant part of the normal variability.

Inconsistencies

In contrast to the data for A elements, there are now several examples in which MARs increase expression but do not produce copy number dependence. The first such observation was the report by Allen et al., (1993) describing the effect of a MAR contained in the yeast ARS-1 element. Tobacco suspension culture cells stably transformed by microprojectile bombardment showed approximately 25 fold higher expression per gene copy when the 35S::GUS reporter construct was flanked by MARs than when it was not. However, the increase in expression was not copy number dependent, and in fact expression was maximal in cell lines with relatively few transgene copies.

Similar results were obtained for a strongly binding MAR isolated from the tobacco genome (Allen et al., 1995). The increase in expression was much greater than with the yeast MAR, in excess of 130 fold on a per gene basis. However, maximal expression was still only observed in lines with low copy numbers.

Poljak et al., (1994) tested "minimal" Drosophila MARs chosen to minimize the chance that they contained any other regulatory information. CAT reporter gene constructs were tested in either HeLa or L cells. Expression of genes bounded by two MARs was stimulated by 20-40 fold on average, but results from an analysis of individual transformed cell lines were quite similar to those of Allen et al. (Allen et al., 1993) in that high variability persisted in the presence of MARs, and expression was not proportional to copy number.

Kalos and Fournier (1995) tested MARs derived from the human *apoB* domain in transient and stable transfection assays with hepatoma cell lines. In these assays, control constructs were efficiently expressed in transient assays but showed low and variable expression in stable transgenic clones. In contrast, single copy transformants containing constructs flanked by the 5' and 3' *apoB* MARs were expressed at consistently higher levels. Thus, it could be

argued that MARs reduced position effects on expression of single copy transformants. However, multicopy transformants yielded poor expression with or without MARs, indicating that MARs are unable to overcome certain classes of chromosomal influences. These results were interpreted in terms of gene silencing phenomena similar to those invoked by Allen et al., (1993). This point will be discussed further below.

Since a chicken lysozyme MAR (the A element) figured prominently in early reports of copy number dependence, it is of particular interest that its role has recently been questioned. In transgenic mice, deletion of both boundary elements from transgenes containing the entire lysozyme domain causes ectopic expression of the gene, but position-independent expression is retained as long as all of the several cis-regulatory elements in both enhancer complexes are retained (Bonifer et al., 1994). In the same set of experiments, deleting one of the enhancer complexes reduced position independence, even though both boundary elements remained intact. These data on the natural gene in transgenic mice offer a striking contrast to earlier reports of A element effects in transfection experiments with cultured cells. The mouse data also contrast sharply with recent data suggesting that A elements normalize expression of reporter genes in transgenic plants (Mlynárová et al, 1995; Mlynárová et al., 1994).

The reason(s) for these differences remain unclear. However, it is worth noting that transforming either plants or cultured cells involves integrating foreign DNA into the genomes of rapidly dividing cells where the incoming DNA is likely to insert into transcriptionally active chromatin (Herman et al., 1990; Kertbundit et al., 1991). In contrast, transgenic mice are produced by injecting DNA into fertilized oocytes. Because oocytes are transcriptionally inactive at the time of injection (Latham et al., 1992; Henerey et al., 1995), there may be less contrast in the accessibility of potentially active and inactive portions of the genome, and incoming DNA may not be so efficiently targeted to active chromosomal regions. In mice, therefore, transgene expression assays may measure *de novo* activation of genes from a condensed chromosomal environment, whereas in cell cultures and plants transgene expression may be largely a function of the extent to which transcription and RNA accumulation can persist in the face of precesses that lead to gene silencing. If this hypothesis is correct, one might reasonably conclude that MARs play a more important role in resisting silencing than they do in promoting activation.

DO MARS AFFECT GENE SILENCING?

It is well known that expression of the same transgene construct can vary over several orders of magnitude in individual transgenic plants (Dean et al., 1988; Jones et al., 1985; Peach and Velten, 1991). This variability was once thought to arise entirely from genomic

position effects, but recent evidence indicates that one or more 'gene silencing' phenomena are also involved (reviewed by Finnegan and McElroy, 1994; Flavell, 1994; Jorgensen, 1993; Jorgensen, 1995; Kooter and Mol, 1993; Matzke and Matzke, 1993; Matzke et al., 1994; Matzke and Matzke, 1995a; Matzke and Matzke, 1995b). The distinction between position effects and gene silencing phenomena is not always a sharp one. In principle, however, position effects on transgene expression reflect pre-existing features of the insertion site, such as proximity to genomic enhancers and degree of chromatin condensation, while gene silencing usually results from homology-dependent interactions involving the transgene itself (although chromosomal position may influence the severity of these interactions).

Forms of Gene Silencing

As we use the term, gene silencing depends in one way or another on homology between transgenes, between transgenes and endogenous genes, or between flanking genomic sequences and sequences elsewhere in the genome. Thus, it has been termed "homology-dependent gene silencing" (Matzke and Matzke, 1993; Matzke et al., 1994) or "repeat-induced gene silencing" (Assaad et al, 1993). Included under this general heading are *co-suppression* (Napoli et al., 1990; van der Krol et al., 1990; Jorgensen, 1992; Jorgensen, 1993; Jorgensen, 1995), in which both a transgene and a homologous endogenous gene are inactivated, and *trans-inactivation* (Matzke and Matzke, 1993; Matzke et al., 1994; Matzke and Matzke, 1995b; Matzke et al., 1989), in which introduction of a second transgene with homology to a previously introduced transgene leads to silencing of one or both. These two types of silencing usually operate in *trans*, but it is also known that silencing can be induced when multiple transgenes are arranged in *cis*, as in the case of tandem arrays or inverted repeats (Assaad et al., 1993; Hobbs et al., 1993).

The molecular basis for gene silencing remains somewhat mysterious, although evidence is accumulating for at least two different mechanisms. Several recent reports support a post-transcriptional mechanism, especially in cases of co-suppression (e.g., Dehio and Scheell, 1994; Metzlaff et al., Van Blokland et al., 1994), while in other cases silencing seems to occur at the transcriptional level and is frequently correlated with increases in DNA methylation (e.g., Brusslan et al., 1993; Matzke et al., 1989; Matzke et al., 1993; Vaucheret, 1994). Several of the clearest examples of transcriptional silencing seem to be triggered by homology in promoter regions that are not present in transcripts. Thus far, the reported cases of post-transcriptional silencing seem to involve either multicopy transgene insertions or homology between transcribed sequences of a transgene and an endogenous gene (as in co-suppression).

The distinction between transcriptional and post-transcriptional mechanisms is not always clean, however. Even where there is good

evidence for a post-transcriptional component, the inactivation process sometimes leads to increases in transgene DNA methylation reminiscent of those associated with transcriptional inactivation (Smith et al., 1994; Ingelbrecht et al., 1994; Wassenegger et al., 1994). One way to resolve this apparent contradiction would be to postulate that transcription can proceed through methylated DNA. However, it is interesting to also consider the hypothesis that the tissues being examined are heterogeneous in their response, with transcriptional silencing occurring in some cells and post-transcriptional silencing in others. There are as yet no data that address the issue of mechanistic heterogeneity, but there are clear indications that somatic gene silencing events occurring during plant development can produce regular or random patterns of activity and inactivity within an organ or tissue (Barnes, 1990; Napoli et al., 1990; Neuhuber et al., 1994; van der Krol et al., 1990; Vaucheret, 1994).

Both transcriptional and post-transcriptional gene silencing mechanisms find precedent in studies with animal cells. Degradation of aberrant or otherwise untranslatable mRNAs has been observed in several laboratories (e.g., Cheng and Maquat, 1993; Pulak and Anderson, 1993), while chromatin condensation and DNA methylation are commonly observed in diverse examples of gene inactivation ranging from X chromosome inactivation and imprinting in mammals to silencing of the HML and HMR mating type genes in yeast (Rivier and Pillus, 1994). In *Drosophila,* position effect variegation (PEV) occurs when chromosomal rearrangements place genes in the vicinity of heterochromatic regions. Such genes frequently become part of the adjacent heterochromatin and are thus inactivated, as described by Henikoff elsewhere in this volume. Of particular interest is the recent observation that mechanisms genetically similar to those that induce heterochromatinization in PEV can also inactivate transgenes at ectopic locations far removed from any large blocks of heterochromatin (Dorer and Henikoff, 1994). Transgenes present in multiple copies are frequently subject to inactivation in this system, especially when arranged as inverted repeats - a result that closely parallels current observations on plant transgenes.

Some informative exceptions to the rule that only multicopy or homologous genes are subject to silencing have been described by Peter Meyer and his colleagues (Meyer and Heidmann, 1994; Meyer et al., 1993; Pröls and Meyer, 1992). The Al gene of maize, with no known sequence homology to any other sequence in Petunia was shown to undergo inactivation even in single copy transformants. However, inactivation may well depend on the homology between sequences near the insertion site and similar sequences elsewhere in the genome, as inactivation was more frequent when the transgene integrated into repetitive DNA.

Each of the several different types of gene silencing can be viewed as one example of a general tendency toward epigenetic inactivation of repeated sequences. It is likely that similar mechanisms account for natural phenomena such as paramutation and

imprinting in maize (Brink, 1973; Kermicle, 1978; Patterson et al., 1993), cytokinin habituation in tobacco (Meins, 1989), and the activity phase changes often observed in transposon studies (Federoff, 1989; Martienssen et al., 1990). Similarly, as originally suggested by Waddington (Waddington, 1953), there are a variety of developmental phase transitions that seem to require epigenetic changes in gene activity (Poethig, 1990). Thus, the phenomena revealed by transgene studies, originally viewed as annoying obstacles to the application of molecular biotechnology, may provide keys to understanding previously obscure gene control mechanisms of fundamental importance in plant evolution and development.

MARs and Silencing

The MAR effects we and others have studied are largest in situations that should strongly favor gene silencing interactions. Both in plants and in animals, increases in gene expression are most prominent in direct transformation experiments that produce multicopy inserts. In most cases, multicopy transformants prepared in this way are expected to contain complex arrays of transgenes at single locus, an arrangement that would facilitate either pairing between transgenes or interactions among their transcripts that would lead to transcriptional or post-transcriptional gene silencing, respectively.

Figure 5 shows that the MAR effects we observe are most prominent in low copy number transformants. No comparable data are available for other plant systems, and the early work with animal cell transfection did not show a similar trend, as noted above. However, recent work with animal cell transfection has also provided examples in which the effect is maximal in low copy number transformants (Poljak et al., 1994). All the available data so far are compatible with an hypothesis suggesting that MARs can resist homology-dependent gene silencing, for example by preventing pairing between multiple copies of the transgene or otherwise affecting the properties of transgene chromatin or the localization of transgenes in the nucleus. To account for the decline in expression at high copy number, we assume that as transgene copy numbers increase it becomes increasingly unlikely that MARs will be able to completely suppress all possible pairing interactions, and that pairing leads to chromatin structure changes that silence not only paired genes but other genes in their immediate vicinity.

The transgenic plant data on MAR effects are also consistent with the idea that MARs primarily reduce gene silencing. As noted above, the magnitude of the increase in expression observed in these experiments is somewhat variable but never more than a few fold, in contrast to the much larger effects seen in cell lines. Where large numbers of transformants have been analyzed, however, it can be seen that one of the major effects of MARs is a reduction in the number of transgenic individuals with very low reporter gene

expression (Mlynárová et al., 1995; Mlynárová et al., 1994). As noted above, it seems likely that many insertion events are eliminated from

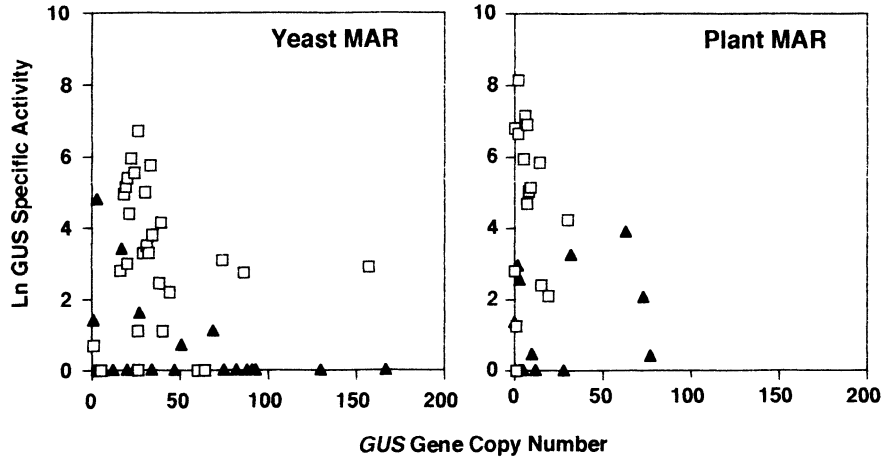

Figure 5. Effects of yeast and plant MARs on *GUS* gene expression following transformation by microprojectile bombardment. Each point represents a cell line established from an independent transformant. Cell lines containing MAR constructs are represented by open squares, and control lines by closed triangles. *GUS* copy numbers were estimated by a combination of quantitative PCR and Southern hybridization analysis. The data are plotted as natural logarithms to more effectively display the variance at low expression levels. For the plant MAR experiment, not all the control data points are visible, even in this log transformation. The full data set contains 16 points for the control and 17 for the MAR construct.

these populations by the selection for a linked drug resistance marker that is inherent in *Agrobacterium* transformation procedures. This selection almost certainly truncates the populations and minimizes any difference in reporter gene activity, such as that attributable to the presence or absence of MARs. However, even after eliminating most of the non-expressors from the starting population in this way, there are still individual that fail to express the reporter gene at high levels. These individuals may represent a subgroup in which the transgenes underwent silencing later in development, after drug selection was removed, or in which the reporter gene has been silenced but the selectable marker is still expressed.

SUMMARY

From the data available thus far, it is reasonable to assume that MARs will soon be included as useful modifiers of gene expression in many of the transgenic plants produced for research and commercial purposes. Their ability to increase gene expression is most dramatically seen in work involving direct DNA transformation, but their ability to reduce the number of low expressors even in populations produced by *Agrobacterium* transformation may still be of considerable importance both in plant breeding and in research applications, such as promoter analysis, in which one must make quantitative comparisons between different gene constructs. It is also possible that MARs will affect generational stability of gene expression, reducing the incidence of transgene silencing in advanced generations or upon crossing into a different genetic background. There are as yet very few data in the public domain that bear on this question (see Finnegan and Mcelroy, 1994; Neuhuber et al., 1994), although significant levels of transgene instability could affect many potential applications.

At present, it seems most logical to think of MARs primarily as increasing gene expression rather than as reducing position effect variation. In addition, it appears likely that their effect on expression most often results from their ability to somehow resist gene silencing. As yet, it is impossible to specify a detailed molecular mechanism for MAR effects, although it is attractive to speculate that in one way or another they may resist chromatin condensation. The latter point might prove especially important for artificial transgene constructs in which the distance between MARs is quite small, as the resulting "mini-domain" might be too small to undergo effective condensation (Allen et al., 1995).

In future studies it will be of obvious interest to characterize more MARs, to continue characterizing the proteins that can bind to them, and to further elucidate the structure of the nuclear matrix. Very little such work has yet been done on plant systems, and it will be of interest to determine the extent to which these basic elements of subnuclear structure differ from those in other organisms.

Future studies will also be required to determine whether MARs can affect both transcriptional and post-transcriptional forms of gene silencing, and to learn more about the molecular mechanisms underlying their effects. In particular, it will be of interest to determine whether or not the MAR effects on gene expression that have been documented in plant and animal systems result from a physical association with to the nuclear matrix. Since we know that exogenous DNA can bind to the matrix during its isolation, even the "endogenous" assay does not permit us to specify whether any particular MAR is bound or not bound *in vivo.* Resolving this question is likely to require a combination of careful structure/function studies for both gene expression and binding, together with structural analyses including transgene and transcript localization studies. Such

studies should also contribute to an understanding of structural constraints on gene expression and the interactions that lead to gene silencing.

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REFERENCES

Adachi, Y., Käs, E., and Laemmli, U.K., 1989, Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions., *EMBO J.* 8:3997.

Allen, G.C., Hall, G., Michalowski, S., Newman, W., Spiker, S., Weissinger, A.K., and Thompson, W.F., 1995, High level transgene epxression in plant cells: Effects of a strong SAR from tobacco, submitted.

Allen, G.C., Hall, G.E., Childs, L.C., Weissinger, A.K., Spiker, S., and Thompson, W. F., 1993, Scaffold attachment regions increase reporter gene epxression in stably transformed plant cells, *Plant Cell* 5: 603.

Amati, B.B., and Glasser, S.M., 1988, Chromosomal ARS and CEN elements bind specifically to the yeast nuclear scaffold, *Cell* 54:967.

Anderson, H.J., and Roberge, M., 1992, DNA topoisomerase II: A review of its involvement in chromosome structure, DNA replication, transcription and mitosis, *Cell Biol. Int. Rep.* 16:717.

Assaad, F.F., Tucker, K.L., and Signer, E.R., 1993, Epigenetic Repeat-Induced Gene Silencing (RIGS) in Arabidopsis, *Plant Mol. Biol.* 22:1067.

Barnes, W.M., 1990, Variable patterns of expression of luciferase in transgenic tobacco leaves, *Proc. Natl. Acad. Sci. USA* 87:9183.

Bevin, A., Guan, Y.H., Peart, J., Cooper, C., and Shaw, P., 1991, Monoclonal antibodies to plant nuclear matrix reveal intermediate filament-related components within the nucleus, *J. Cell Sci.* 98:293.

Bode, J., Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C., and Kohwi-Shigematsu, T., 1992, Biological significance of unwielding capability of nuclear matrix-associating DNAs, *Science* 255:195.

Bode, J. and Maass, K., 1988, Chromatin domain surrounding the human interferon-a gene as defined by scaffold-attached regions, *Biochemistry* 27:4706.

Bonifer, C., Hecht, A., Saueressig, H., Winter, D.M., and Sippel, A.E., 1991, Dynamic chromatin: The regulatory domain organization of eukaryotic gene loci, *J. Cell. Biochem.* 47:99.

Bonifer, C., Vidal, M., Grosveld, F., and Sippel, A., 1990, Tissue specific and position independent expression of the complete gene domain for chick lysozyme in transgenic mice, *EMBO J.* 9:2843.

Bonifer, C., Yannoutsos, N., Krüger, G., Grosveld, F., and Sippel, A.E., 1994, Dissection of the locus control function located on the chicken lysozyme gene domain in transgenic mice, *Nucleic Acids Res.* 22:4202.

Boulikas, T., 1993, Nature of DNA sequences at the attachment regions of genes to the nuclear matrix., *J. Cell. Biochem.* 52:14.

Breyne, P., Van Montagu, M., Depicker, A., and Gheysen, G., 1992, Characterization of a plant scaffold attachment region in a DNA fragment that normalizes transgene expression in tobacco, *Plant Cell* 4:463.

Brink, R.A., 1973, Paramutation, *Annu. Rev. Genet.* 7: 129-152.

Brusslan, J.A., Karlin-Neuman, G.A., Huang, L., and Tobin, E. M., 1993, An *Arabidopsis* mutant with a reduced level of *cab140* RNA is a result of cosuppression, *Plant Cell* 5:667.

Capco, D.G., Wan, K.M., and Penman, S., 1982, The nuclear matrix: three-dimensional archetecture and protein composition, *Cekk* 29:847.

Cheng, J., and Maquat, L.E., 1993, Nonsense condons can reduce the abundance of nuclear mRNA without affecting the abundance of pre-mRNA or the half life of cytoplasmic mRNA, *Mol. Cell. Biol.* 13:1892.

Christou, P., Swain, W.F., Yang, N.S., and McCabe, D.E., 1989, Inheritance and expression of foreign genes in transgenic soybean plants, *Proc. Natl. Acad. Sci. USA* 86:7500.

Conkling, M.A., Cheng, C.-L., Yamamoto, Y.T., and Goodman, H.M., 1990, Isolation of transcriptionally regulated root-specific genes from tobacco, *Plant Physiol.* 93:1203.

Dean, C., Jones, J., Favreau, M., Dunsmuir, P., and Bedbrook, J., 1988, Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants, *Nucleic Acids Res.* 16:9267.

Dehio, C., and Schell, J., 1994, Identification of plant genetic loci involved in a posttranscriptional mechanism for meiotically reversible gene silencing, *Proc. Natl. Acad. Sci. USA* 91:5538.

Dickinson, L.A., Joh, T., Kohwi, Y., and Kohwi-Shigematsu, T., 1992, A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition, *Cell* 70:631.

Dorer, D.R., and Henikoff, S., 1994, Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila, *Cell* 77:993.

Dworetsky, S.I., Wright, K.L., Fey, E.G., Penman, S., Lian, J. B., Stein, J. L., and Stien, G.S., 1992, Sequence-specific DNA binding proteins are components of a nuclear matrix-attachment site, *Proc. Natl. Acad. Sci. USA* 89:4178.

Earnshaw, W.C., and Heck, M.M.S., 1985, Localization of topoisomerase II in mitotic chromosomes, *J. Cell. Biol.* 100:1716.

Earnshaw, W.C., and Heck, M.M.S., 1988, The ultrastructure of the mitotic chromosome scaffold: studies using whole-mount electron microscopy and immunocytological techniques, *in:* "Chromosomes and Chromatin," K. W. Adolph, ed., CRC Press.

Farache, G., Razin, S.V., Rzeszowska-Wolney, J., Moreau, J., Fecillas Targa, F., and Scherrer, K., 1990, Mapping of structural and transcription-related matrix attachment sites in the b-globin gene domain of avian erythroblasts and erythrocytes., *Mol. Cell. Biol. 10*:5349.

Federoff, N.V., 1989, About maize transposable elements and development, *Cell* 56:181.

Ferraro, A., Grandi, P., Eufemi, M., Altieri, F., and Turano, C., Cross-linking of nuclear proteins to DNA by cis-Diamminedichloroplatinum in intact cells-involvement of nuclear matrix proteins, *FEBS Lett.* 307:383.

Finnegan, J., and McElroy, D., 1994, Transgene inactiviation: Plants fight back!, *Biotechnology* 12:883.

Flavell, R.B., 1994, Inactivation of gene expression in plants as a consequence of specific sequence duplication, *Proc. Natl. Acad. Sci. USA* 91:3490.

Gasser, S., and Laemmli, U., 1986, Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of D. melanogaster, *Cell* 46:521.

Gasser, S.M., Amati, B.B., Cardenas, M.E., and Hofmann, J.F.-X., 1989, Studies on scaffold attachment sites and their relation to genome function, *Int. Rev. Cytol.* 119:57.

Gasser, S.M., and Laemlli, U.K., 1986b, The organization of chromatin loops: characterization of a scaffold attachment site, *EMBO J.* 5:511.

Goldman, M.A., 1988, The chromatin domain as a unit of gene regulation, *BioEssays* 9:50.

Grabber, A., Eberharter, A., Gstraunthaler, G., and Loidl, P., 1992, Characterization of nuclear matrix proteins of Physarum polycephalum and mammalian cells, *Cell Biol. Int. Rep.* 16:1151.

Hall, G., Allen, G.C., Loer, D.S., Thompson, W.F. and Spiker, S., 1991, Nuclear scaffolds and scaffold attachment regions (SARS) in higher plants, *Proc. Natl. Acad. Sci. USA* 88:9320.

Hall, G., Jr., and Spiker, S., 1994, Isolation and characterization of nuclear scaffolds, *in*: "Plant Molecular Biology Manual," S. B. Gelvin and Schilperoort, R. A., ed., Kluwer Academic Publishers, Dordrecht, The Netherlands.

Henerey, C.C., Miranda, M., Wiekowski, M., Wilmut, I., and DePamphilis, M. L., 1995, Repression of gene expression at the beginning of mouse development, Devel. Biol. 169:448.

Herman, L., Jacobs, A., Van Montagu, M., and Depicker, A., 1990, Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events, *Mol. Gen. Genet.* 224:248.

Hobbs, S.L.A.., Warkentin, T.D., and DeLong, C.M.O., 1993, Transgene copy number can be positively or negatively associated with transgene epxression, *Plant Mol. Biol.* 21:17.

Huber, M., Bosch, F.X., Sippel, A.E., and Bonifer, C., 1994, Chromosomal position effects in chicken lysozyme gene transgenic mice are correlated with suppression of hypersensitive site formation, *Nucleic Acids Res.* 22:4195.

Ingelbrecht, I., Van Houdt, H., Van Montagu, M., and Depicker, A., 1994, Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation, *Proc. Natl. Acad. Sci. USA* 91:10502.

Ivanchenko, M., and Avramova, Z., 1992, Interaction of MAR-sequences with nuclear matrix proteins, *J. Cell Biochem.* 50:190.

Ivanchenko, M., Tasheva, B., Stoilov, L., Christova, R., and Zlatanova, J., 1993, Characterization of some nuclear matrix proteins in maize, *Plant Sci.* 91:35.

Izaurralde, E., Käs, E., and Laemmli, U. K., 1989, Highly preferential nucleation of histone H1 assembly on scaffold-associated regions, *J. Mol. Biol.* 210:573.

Jack, R.S., and Eggert, H., 1992, The elusive nuclear matrix, *Eur. J. Biochem.* 209:503.

Jarmon, A.P., and Higgs, D. R., 1988, Nuclear scaffold attachment sites in the human globin gene complexes, *EMBO J.* 7:3337.

Jones, J.D.G., Dunsmuir, P., and Bedbrook, J., 1985, High level expression of introduced chimaeric genes in regenerated transformed plants, *EMBO J.* 4:2411.

Jorgensen, R., 1992, Silencing of plant genes by homologous transgenes, *AgBiotech News and Information* 4:265N.

Jorgensen, R., 1993, The germinal inheritance of epigenetic information in plants, *Phil. Trans. Roy. Soc. Lond. B* 339:173.

Jorgensen, R.A., 1995, Cosuppression, flower color patterns, and metastable gene expression states, *Science* 268:686.

Kalos, M., and Fournier, R.E.K., 1995, Position-independent transgene expression mediated by boundary elements from the apolipoprotein B chromatin domain, *Mol. Cell. Biol.* 15:198.

Kermicle, J., 1978, Imprinting of gene action in maize endosperm, *in*: "Maize Breeding and Genetics," D. B. Walton, ed., Wiley-Interscience, NY.

Kertbundit, S., De Greve, H., Deboeck, F., Van Montagu, M., and Hernalsteens, J.-P., 1991, *In vivo* random ß-glucuronidase gene fusions in *Arabidopsis thaliana, Proc. Natl. Acad. Sci. USA* 88:5212.

Klehr, D., Maass, K., and Bode, J., 1991, Scaffold-attached regions from the human interferon ß domain can be used to enhance the stable expression of genes under the control of various promoters, *Biochemistry* 30:1264.

Kooter, J.N., and Mol, J. N.M., 1993, Trans-inactivation of gene expression in plants, *Curr. Opinion Biotechnol.* 4:166.

Krachmarov, C., Stoilov, L., and Zlatanova, J., 1991, Nuclear matrices from transcriptionally active and inactive plant cells, *Plant Science* 76:35.

Latham, K.E., Stolter, D., and Schultz, R., 1992, Acquisition of a transcriptionally permissive state during the 1-cell stage of mouse embyrogenesis, Develop. Biol. 149:457.

Levy-Wilson, B., and Fortier, C., 1989, The limits of the DNase I sensitive domain of the human apolipoprotein B gene coincide with the locations of

chromosomal anchroage loops and define the 5' and 3' boundaries of the gene, *J. Biol. Chem.* 264:21196.
Martienssen, R., Barkan, A., Taylor, W.C., and Freeling, M., 1990, Somatically heritable switches in the DNA modification of *Mu* transposable elements monitored with a suppressible mutant in maize, *Genes and Dev.* 4:331.
Matzke, M., and Matzke, A.J.M., 1993, Genomic imprinting in plants: Parental effects and *trans*-inactivation phenomena, *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 44:53.
Matzke, M., Matzke, A.J.M., and Mittelsten Scheid, O., 1994, Inactivation of repeated genes -- DNA-DNA interaction? *in:* "Homologous Recombination and Gene Silencing in Plants," J. Paszkowski, ed., Kluwer Academic Publishers, Amsterdam.
Matzke, M.A., and Matzke, A.J.M., 1995a, Homology-dependent gene silencing in transgenic plants: What does it really tell us?, *Trends Genet* 11:1.
Matzke, M.A., and Matzke, A.J.M., 1995b, How and why do plants inactivate homologous (trans)genes?, *Plant Physiol.* 107:679.
Matzke, M.A., Neuhuber, F., and Matzke, A.J.M., 1993, A variety of epistatic interactions can occur between partially homologous transgene loci brought together by sexual crossing, *Mol. Genl. Genet.* 236:379.
Matzke, M.A., Primig, M., Trnovsky, J., and Matzke, A.J.M., 1989, Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants, *EMBO J.* 8:643.
McCabe, D.E., Swain, W.F., Martinell, B.J., and Christou, P., 1988, Stable transformation of soybean (Glycine max) by particle accerlation, *Biotechnology* 6:923.
McKnight, R.A., Shamay, A., Sankaran, L., Wall, R.J., and Hennighausen, L., 1992, Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice, *Proc. Natl. Acad. Sci. USA* 89:6943.
McNulty, A.K., and Saunders, M.J., 1992, Purification and immunological detection of pea nuclear intermediate filaments - evidence for plant nuclear lamins, *J. Cell Sci.* 103:407.
Meins, F., 1989, Habituation: heritable variation in the requirement of cultured plant cells for hormones, *Ann. Rev. Genet.* 32:395.
Metzlaff, M., O'Dell, M., and Flavell, R.B., 1995, Suppression of chalcone synthase A activities in Petunia by the addition of a transgene encoding chalcone synthase, *in*: "Gene Silencing," D. Grierson, ed., Plenum Press, NY, in press.
Meyer, P., and Heidmann, I., 1994, Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreigh DNA, *Mol. Gen. Genet.* 243:390.
Meyer, P., Heidmann, I., and Niedenhof, I., 1993, Differences in DNA-methylation are associated with a paramutation phenomenon in transgenic petunia, *Plant J.* 4:89.
Mirkovitch, J., Mirault, M.-E., and Laemmli, U.K., 1984, Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold, *Cell* 39:223.
Mlynárová, L., Jansen, R.C., Conner, A.J., Stiekema, W.J., and Nap, J.-P., 1995, The MAR-meidated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants, *Plant Cell* 7:599.
Mlynárová, L., Loonen, A., Heldens, J., Jansen, R.C., Keizer, P., Stiekema, W.J., and Nap, J.-P., 1994, Reduced position effect in mature transgenic plants conferred by the chick lysozyume matrix-associated region, *Plant Cell* 6:417.
Napoli, C., Lemieux, C., and Jorgensen, R., 1990, Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans, Plant Cell* 2:279.
Nelson, H.C. M., Finch, J.T., Luisi, B.F., and Klug, A., 1987, The structure of an oligo(dA)_oligo(dT) tract and its biological implications, *Nature* 330:221.
Neuhuber, F., Park, Y.-D., Matzke, A.J.M., and Matzke, M.A., 1994, Susceptibility of transgene loci to homology-dependent gene silencing, *Mol. Gen. Genet.* 244:230.

Paranjape, S.M., Kamakaka, R.T., and Kadonaga, J.T., 1994, Role of chromatin structure in the regulation of transcription by RNA polymerase II, *Ann. Rev. Biochem.* 63:265.

Patterson, G., Thorpe, C.J., and Chandler, V.J., 1993, Paramutation, an allelic interaction, is associated with a stable and heritable reduction of transcription of the maize *b* regulatory gene, *Genetics* 135:881.

Paulson, J.R., and Laemmli, U.K., 1977, The structure of histone-depleted metaphase chromosomes, *Cell* 12: 817.

Peach, C., and Velten, J., 1991, Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promotrs, *Plant Mol. Biol* 17:49.

Peng, J.Y., Wen, F.J., Lister, R.L., and Hodges, T.K., 1995, Inheritance of *gusA* and *neo* genes in transgenic rice, *Plant Mol. Biol* 27:91.

Phi-van, L., and Strätling, W.H., 1988, The matrix attachment regions of the chick lysozyme gene co-map with the boundaries of the chromatin domain, *EMBO J.* 7:655.

Phi-Van, L., von Kries, J.P., Ostertag, W., and Strätling, W.H., 1990, The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes, *Molec. Cell. Biol.* 10:2302.

Poethig, R.S., 1990, Phase change and the regulation of shoot morphogenesis in plants, *Science* 250:923.

Poljak, L., Seum, C., Mattioni, T., and Laemmli, U.K., 1994, SARs stimulate but do not confer position independent gene expression, *Nucleic Acids Res.* 22:4386.

Pröls, F., and Meyer, P., 1992, The methylation patterns of chromosomal integration regions influence gene activity of transferred DNA in Petunia hybrida, *Plant J.* 2:465.

Pulak, R., and Anderson, P., 1993, mRNA surveillance by the *Caenorhabditis elegans smg* genes, *Genes and Develop.* 7:1885.

Reeves, R., 1984, Transcriptionally active chromatin, *Biochem. Biophys. Acta* 782:343.

Register, J.C., Peterson, D.J., Bell, P.J., Bullock, W.P., Evans, I.J., Frame, B., Greenland, A.J., Higgs, N.S., Jepson, I., Jiao, S., Lewnau, C.J., Sillick, J.M., and Wilson, M.H., 1994, Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bomardment, *Plant Mol. Biol.* 25:951.

Rivier, D.H., and Pillus, L., 1994, Silencing speaks up, *Cell* 76:963.

Romig, H., Fackelmayer, F.O., Renz, A., Ramsperger, U., and Richter, A., 1992, Characterization of SAF-A, a novel nuclear DNA binding protein from HeLa cells with high affinity for nuclear matrix/scaffold attachment DNA elements, *EMBO J.* 11:3431.

Schöffl, F., Schröder, G., Kleim, M., and Rieping, M., 1993, An SAR sequence containing 395 bp DNA fragment mediates enhanced, gene-dosage-correlated expression of a chimaeric heat shock gene in transgenic tobacco plants, *Transgenic Res.* 2:93.

Smith, H.A., Swaney, S.L., Parks, T.D., Wernsman, E.A., and Dougherty, W.G., 1994, Transgenic plant virus resistance mediated by untranslatable sense RNAs: Expression, regulation, and fate of nonessential RNAs, *Plant Cell* 6:1441.

Stalder, J., Larsen, A., Engel, J.D., Dolan, M., Groudine, M., and Weintraub, H., 1980, Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNAaseI, *Cell* 20:451.

Stief, A., Winter, D.M., Strätling, W.H., and Sippel, A.E., 1989, A nuclear DNA attachment element mediates elevated and position-independent gene activity, *Nature* 341:343.

Tsutsui, K., Tsutsui, K., Okada, S., Watarai, S., Seki, S., Yasuda, T., and Shohmori, T., 1993, Identification and characterization of a nuclear scaffold protein that binds the matrix attachment region DNA, *J. Biol. Chem.* 268:12886.

Van Blokland, R., Van der Geest, N., Mol, J.N.M., and Kooter, J.M., 1994, Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover, *Plant J.* 6:861.

van der Geest, A.H.M., Hall, G.E., Spiker, S., and Hall, T.C., 1994, The beta-phaseolin gene is flanked by matrix attachment regions, *Plant J.* 6:413.
van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitje, A.R., 1990, Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression, *Plant Cell* 2:291.
Vaucheret, H., 1994, Promoter-dependent trans-inactivation in transgenic tobacco plants: kinetic aspects of gene silencing and gene reactivation, *C. R. Acad. Sci. Paris* 317:310.
von Kries, J.P., Buhrmester, H., and Strätling, W.H., 1991, A matrix/scaffold attachment region binding protein-identification, purification and mode of binding, *Cell* 64:123.
Waddington, C.H., 1953, Epigenetics and evolution, *Symp. Soc. Exp. Biol.* 7:186.
Wassenegger, M., Heimes, S., Riedel, L., and Sänger, H. L., 1994, RNA-directed de novo methylation of genomic sequences in plants, *Cell* 76:567.
Weintraub, H., and Groudine, M., 1976, Chromosomal subunits in active genes have and altered conformation, *Science* 193:848.
Zhao, K., Käs, E., Gonzalez, E., and Laemmli, U., 1993, SAR-dependent mobilization of histone H1 by HMG-I/Y *in vitro*: HMG-I/Y is enriched in H1-depleted chromatin, *EMBO j.* 12:3237.