Characterization of a Plant Scaffold Attachment Region in a DNA Fragment That Normalizes Transgene Expression in Tobacco

Peter Breyne, Marc Van Montagu, Ann Depicker, and Godelieve Gheysen
Laboratorium voor Genetica, Universiteit Gent, B-9000 Gent, Belgium

Using a low-salt extraction procedure, we isolated nuclear scaffolds from tobacco that bind specific plant DNA fragments in vitro. One of these fragments was characterized in more detail; this characterization showed that it contains sequences with structural properties analogous to animal scaffold attachment regions (SARs). We showed that scaffold attachment is evolutionarily conserved between plants and animals, although different SARs have different binding affinities. Furthermore, we demonstrated that flanking a chimeric transgene with the characterized SAR-containing fragment reduces significantly the variation in expression in series of transformants with an active insertion, whereas a SAR fragment from the human β-globin locus does not. Moreover, the frequency distribution patterns of transgene activities showed that most of the transformants containing the plant SAR fragment had expression levels clustered around the mean. These data suggest that the particular plant DNA fragment can insulate the reporter gene from expression-influencing effects exerted from the host chromatin.

INTRODUCTION

The chromatin in the eukaryotic interphase nucleus needs to be highly ordered and compacted to ensure accuracy of nuclear processes such as replication and transcription. Results of electron microscopy and sedimentation analyses support the hypothesis that the 30-nm chromatin fiber is organized in looped domains through interactions with a proteinaceous structure (reviewed by Paulson, 1988). This structure has been called the nuclear scaffold (Mirkovitch et al., 1984) or the nuclear matrix (Berezney and Coffey, 1974), depending on the extraction method used. Nuclear scaffolds are retained after extraction with a mild detergent, lithium diiodosalicylate (LIS), at physiological salt concentrations, whereas nuclear matrices are obtained after high-salt extraction.

The chromatin loops most probably interact with this scaffold or nuclear matrix through scaffold attachment regions (SARs) or matrix-associated regions, respectively. SARs are DNA sequences that remain specifically associated with the scaffold after digestion of the chromatin with restriction enzymes. Such attachment sites have been identified in the genomic DNA of Drosophila, several vertebrates, and yeast (Gasser et al., 1989; Hofmann et al., 1989; Stief et al., 1989; Amati and Gasser, 1990). SARs are usually 300- to 500-bp long, possess an A+T content of >70%, and are found exclusively in noncoding sequences. Because they are only homologous in a number of short DNA motifs (Gasser and Laemmli, 1986a, 1986b; Amati and Gasser, 1990; Amati et al., 1990), they do not cross-hybridize to each other. The ability of SARs to bind scaffolds from other species, however, demonstrates that they are functionally conserved during evolution (Cockerill and Garrard, 1986a, 1986b; Amati and Gasser, 1988, 1990).

The function of SARs in vivo is not very clear. Farache et al. (1990) reported on a difference between structural and transcription-related SARs in the chicken α-globin gene cluster. Earlier reports already showed that certain SARs are adjacent to or coincide with enhancer elements, whereas others form boundaries of nuclease-sensitive domains (reviewed by Gasser et al., 1989). In yeast, it has been shown that autonomously replicating sequences and centromeres are bound to the nuclear scaffold (Amati and Gasser, 1988). Another possible function of SARs is to enhance gene expression and/or normalize it by protection against position effects. For example, the SAR of the chicken lysozyme gene promotes high-level and less position-dependent gene expression in both a homologous and a heterologous background (Stief et al., 1989; Phi-Van et al., 1990).

Nuclear matrices isolated from plants (onion, Moreno Díaz de la Espina et al., 1991; carrot, Beven et al., 1991) show morphological similarities to those of other eukaryotes. The protein pattern of the matrices is complex, and preliminary results showed the presence of polypeptides related to intermediate filament proteins (Beven et al., 1991). Two plant SARs were identified by in vitro binding of plant DNA to animal scaffolds (Izaurralde et al., 1988; Mielke et al., 1990), and, more recently,
the isolation of nuclear scaffolds from tobacco allowed the identification of SARs in the 3' flanking region of three root-specific tobacco genes (Hall et al., 1991).

To study the structural and possible functional properties of SAR elements in plants in more detail, we isolated scaffolds from tobacco and investigated the specific interaction of plant DNA fragments and animal SARs with these scaffolds. Furthermore, we analyzed more than 50 transgenic tobacco clones containing a reporter gene flanked by two different SAR-containing fragments and showed that the fragment with a plant SAR reduces variation in transgene expression almost 20-fold.

RESULTS

Isolation of Nuclear Scaffolds from Tobacco

We established an extraction procedure for tobacco scaffolds based on the low-salt method described by Mirkovitch et al. (1984). To isolate tobacco scaffolds, we modified the procedure at certain steps (see Methods) for two reasons. First, scaffolds need to be extracted from very pure nuclei, and plant nuclear preparations are often contaminated with cell wall fragments that seem to inhibit DNA restriction. Therefore, we compared different purification methods and found that isolating the nuclei from protoplasts and purifying them on Percoll gradients resulted in nuclei that were structurally well shaped and functionally intact when assayed by run-on experiments. Second, in animal systems, stabilization of the nuclei prior to scaffold extraction is necessary to prevent solubilization of the scaffold proteins (Izaurralde et al., 1988). Figure 1 shows a comparison of scaffolds extracted from nuclei stabilized at 42°C in the presence of 0.5 mM Cu^{2+} and from unstabilized nuclei. Surprisingly, there are no major differences in protein composition of both scaffold preparations (Figure 1), and both have the same DNA binding properties (data not shown).

In Vitro Binding of Plant DNA Fragments to Tobacco Scaffolds

For the identification of plant SARs, we used the clone L4p1 (Okamuro et al., 1986) that contains the 17.1-kb insert shown in Figure 2C. This DNA fragment from the soybean genome harbors the seed-specific lectin gene and four other genes with different tissue specificities. Because this large clone contains differently regulated genes, it was not unlikely that it might contain one or more SARs. Preliminary evidence for the presence of SARs in the L4p1 clone was obtained in a binding experiment to Drosophila scaffolds. After cutting with HindIII, EcoRI, and XhoI, two vector and seven plant DNA fragments were obtained (Figure 2C), and the attachment pattern revealed that five of the seven plant DNA fragments were more or less retained in the scaffold fraction (data not shown).

In a next step, L4p1 was used in a binding assay with tobacco scaffolds. The fragments recovered in the supernatant versus pellet fraction were the same as those with Drosophila scaffolds, and the binding affinities were only slightly different. As shown in Figure 2A, the two vector fragments and a 1.25-kb plant DNA fragment are restricted to the supernatant. Four other fragments (5.8, 3, 1.55, and 0.8 kb) are retained in the scaffold fraction. The 2.3-kb band contains two fragments, of which one is attached (see below). Figure 2B presents a more precise SAR mapping. Two EcoRI-BamHI fragments (Figure 2C, L4L and L4R) covering the entire 17.1-kb insert were isolated. L4L digested with BglIII, XbaI, and KpnI (Figure 2B, section 1) has a 0.6-kb EcoRI-BglIII fragment (S1), a 1-kb BglIII fragment (S2), and a 1.3-kb BglIII-KpnI (S3) fragment that are scaffold attached. The S2 fragment could be delimited further to a 0.7-kb BglIII-HindIII associated region (Figure 2C). L4R digested with BglIII, XbaI, and Hpal (Figure 2B, section 2) has 0.6-kb BamHI-XbaI (S4a) and 1-kb XbaI-BglIII (S4b) attached fragments that both contain a part of the same SAR (Figure 2C, S4). Two other SARs are harbored on a 2.1-kb XbaI-Hpal (S5) fragment and a 1.1-kb BglIII-EcoRI (S6) fragment.

In summary, the 17.1-kb plant DNA region contains six SAR elements as defined by our in vitro binding assays, and, except for S6, all lie between the transcribed regions (Figure 2C).
Structural Properties of a Plant SAR

The 1.55-kb EcoRI-HindIII fragment carrying S1 was named the P1-SAR fragment and further analyzed. Sequence analysis revealed a 520-bp region that has an A+T content of 78% and another smaller region of 190 bp with an A+T content of 70%. Both contain several of the sequence motifs present in animal SARs. As shown in Figure 3A, these include A-boxes (AATAAA) and T-boxes (TTATTTT) (Gasser and Laemmli, 1986b), large poly(dAdT) stretches, and the sequence ATATTT (or AAATAT) (Mielke et al., 1990). These motifs are only sparsely present in the rest of the 1.55-kb fragment. In contrast, poly(dA) or poly(dT) stretches are scattered throughout the fragment. For binding assays, we used the P1-SAR fragment cloned 3' of the β-glucuronidase (gusA) reporter gene in the vector pNGUS (see Methods; Figure 3A). The 3' octopine synthase (ocs) gene region in this plasmid contains a 400-bp sequence that has an A+T content of ~70% (Figure 3A) and could therefore serve as an internal control in our binding experiments. To eliminate the possibility that the length of the AT-rich fragment would determine binding specificity, the 3' ocs region was cut out as a 1.77-kb BamHI-EcoRV fragment.

Figure 3B shows the result of the binding experiments. The 1.55-kb P1-SAR fragment binds to the scaffolds, whereas the 1.77-kb 3' ocs fragment as well as the other vector fragments are recovered in the supernatant (Figure 3B, section 2). Cutting the 1.55-kb fragment with Apal prior to binding shows that the 520-bp AT-rich region interacts more efficiently with the scaffolds than does the remaining region of 1.03 kb that attaches only poorly, although it still contains the 190-bp AT-rich region (Figure 3B, section 3). The specific binding affinity of the P1-SAR fragment was measured by adding different concentrations of sheared Escherichia coli DNA (which has an A+T content of at least 50%) during binding assays. P1-SAR attachment gradually decreases with increasing amounts of E. coli DNA, and only at a 10,000-fold excess to the probe is the association abolished (Figure 3B, section 4), indicating that the interaction of the P1-SAR fragment to tobacco scaffolds is specific.
Animal SARs Bind to Tobacco Scaffolds with Different Affinities

That scaffold attachment is conserved between plants and animals is indicated by the fact that the SARs of the L4p1 clone bind to Drosophila scaffolds. Analogous results were also obtained for two other plant DNA fragments (see Introduction). To test whether animal SARs are also able to associate with tobacco scaffolds, both a Drosophila SAR fragment and a human SAR fragment were cloned in the vector pNGUS and tested in in vitro binding assays. For the Drosophila SAR, we used the 5′ SAR fragment from the fushi tarazu (ftz) locus (Gasser and Laemmli, 1988b) that is harbored on a 1.15-kb EcoRI fragment and comaps with one of the enhancer elements of the ftz gene. The human SAR was derived from the β-globin locus. A 2.8-kb EcoRI-BgIII fragment present 5′ upstream from the ε gene was identified as a SAR-containing region by Jarman and Higgs (1988). Figure 4A shows that the ftz-SAR fragment can bind to the scaffolds much better than the β-globin SAR fragment. Incubating tobacco scaffolds with a mixture of the three SARs gave identical results: the P1-SAR and the ftz-SAR fragments bind effectively with the ftz SAR having the highest affinity; on the contrary, nearly all the β-globin SAR fragments remain unattached. This is most probably not due to the length of the fragment (the β-globin SAR fragment is about twice the size of the other two SAR fragments) because the P1-SAR does not lose binding affinity when present on a fragment of the same length (data not shown). Moreover, Figures 2A and 2B show that longer DNA fragments can associate to the same extent as shorter fragments. In an analogous experiment, the same probes were incubated with scaffolds prepared from rat liver nuclei, and no significant differences were observed among the three SARs (data not shown).

Because the P1-SAR fragment and the ftz-SAR fragment show comparable binding capacities, they could occupy the same binding sites on the tobacco scaffolds. Competition experiments with the P1-SAR and the ftz-SAR fragments showed that the ftz-SAR fragment displaces most of the P1-SAR fragment already at a concentration of 20 μg of ftz SAR plasmid DNA per A260 unit of scaffolds (Figure 4B).

Figure 4. Scaffold Attachment of Animal SARs to Tobacco Scaffolds. (A) Binding of the ftz-SAR fragment and the β-globin SAR alone and of a mixture of the P1-SAR fragment with both animal SARs. The SAR fragments are marked with asterisks; the other fragments were derived from the vector pNGUS. Fifty nanograms of labeled SAR plasmids was added to scaffolds prepared from 0.3 A260 units of nuclei, and identical volumes of pellet and supernatant fractions were loaded on gels. For the mixture, equal concentrations of each SAR plasmid were mixed before labeling. The upper asterisk indicates the β-globin SAR fragment; the middle one, the P1-SAR fragment; and the lower one, the ftz-SAR fragment. Due to differential labeling, the P1-SAR is underrepresented. P, pellet; S, supernatant. (B) Competition experiment between the P1-SAR and ftz-SAR fragments both cloned in pGEM2 (Promega). One hundred nanograms of the P1-SAR clone was digested with appropriate enzymes, labeled, and incubated with scaffolds corresponding to 0.3 A260 units of nuclei. Digested, cold ftz-SAR DNA was added at different concentrations, indicated in micrograms beneath lanes 1 to 4, with linearized pGEM2 to keep the total amount of added DNA constant. Only the pellet fractions are shown. s, P1-SAR; T, total probe; v, vector.

Flanking a Reporter Gene with the P1-SAR Fragment Decreases Variation in Gene Expression in Transgenic Plant Tissue

Having identified a plant SAR with specific binding affinity to tobacco scaffolds, we investigated its possible functional role in gene expression in vivo. We constructed T-DNA vectors carrying a selectable hygromycin resistance (hyg) gene and the gusA reporter gene flanked by SAR-containing fragments, as shown in Figure 5. The 1.55-kb P1-SAR, the 2.8-kb β-globin SAR, and the 1.15-kb ftz-SAR fragments were cloned 5′ and 3′ from the reporter gene, resulting in the vectors pNG611, pNG622, and pNG633, respectively. As a control, we used the same T-DNA construct without SARs (pNG6; Figure 5). pNG6, pNG611, and pNG622 showed essentially the same GUS activity when electroporated in tobacco protoplasts, demonstrating that there are no general enhancer or silencer sequences present in the two SAR fragments (data not shown).

The ftz SAR comaps with an enhancer sequence, and transient expression revealed that this enhancer increased the gusA expression severalfold (data not shown); therefore, pNG633 was not included in the following transformation experiments.

The constructs pNG6, pNG611, and pNG622 were introduced into tobacco protoplasts using Agrobacterium (see Methods).
For each construct, 60 hygromycin-resistant calli were tested for GUS activity, and calli lacking detectable GUS activity were omitted from further analysis. Table 1 is a summary of the results displaying the sample size, the mean expression level, the variance, and the coefficient of variation for each series of calli. Table 1 shows that the mean expression is 50% higher in the calli with a pNG622 T-DNA as compared with the pNG6 series. This could indicate that there is a low level of enhancement due to the β-globin SAR fragment. The variance of approximately 10,000 demonstrates that there is an enormous intertransformant variability in gusA expression with the constructs pNG6 and pNG622, a situation that is frequently encountered among different transformants from a population. Calli containing the reporter gene between P1-SARs behave differently. The mean expression level is twofold lower and, interestingly, the variance is 15-fold lower compared with the pNG6 series. However, because the variance is influenced by the mean, it is better to compare the coefficient of variation. This demonstrates that the series with pNG622 already contains less variation than the pNG6 series. Among the pNG611 calli, variation is further reduced to a level that is 80% lower than among pNG6 calli. Both a Bartlett’s test and a log-ANOVA (see Methods) revealed that the variances of pNG6 and pNG622 are homogenous (P < 0.005), whereas the pNG6 and pNG611 variances are heterogeneous (P < 0.005).

At first sight, these data might suggest that flanking the gusA gene with the P1-SAR only results in a silencing effect in stable transformants, diminishing the total variation in the population. That this is not the case can be deduced from the frequency distribution patterns shown in Figure 6. GUS activities from calli containing the constructs without SAR fragments or with the β-globin SAR fragments are randomly distributed over all classes. For the pNG6 series, 40% of the calli have a GUS activity lower than 10 units per milligram and 60% contain intermediate to high activities. Calli with pNG622 are uniformly scattered over all classes. However, pNG611 calli tend to be normally distributed around the mean expression. Ninety percent of the calli are restricted to four classes. There are no calli in the classes of high expression, and low-expressing calli are underrepresented compared to the control series. If there were only a silencing effect in pNG611, more calli would be expected in the class of very low expression (<10 units per milligram). Performing a Mann-Whitney U test showed that the difference in distribution of the pNG6 and pNG611 series is significant (P < 0.001).

### DISCUSSION

**Extraction of Scaffolds from Tobacco Nuclei and Attachment of Plant DNA Fragments**

Based on the method described by Mirkovitch et al. (1984), we have isolated a nuclear substructure from tobacco protoplasts that is analogous to the nuclear scaffolds of animals and yeast. Perhaps the most remarkable difference between scaffold extraction from animal or yeast nuclei and from tobacco is that a heat and/or copper stabilization of the tobacco nuclei prior to LIS extraction is not necessary. In Drosophila, vertebrates, and yeast, LIS extraction of unstabilized nuclei results in solubilization of almost all proteins, except for the nuclear lamins (Izaurralde et al., 1988; Cardenas et al., 1990). Although the mechanism of heat-induced stabilization is unclear, there seems to be a correlation with stress responses. Incubation of intact cells at heat shock temperatures is sufficient for stabilization of the nuclear scaffolds (Gasser et al., 1989; Cardenas et al., 1990). Because nuclei prepared from fresh tobacco leaves gave the same scaffold protein composition as protoplast nuclei (data not shown), it is unlikely that stress during

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**Table 1. Main Statistical Data Circumscribing the Different Series of Calli**

<table>
<thead>
<tr>
<th>Statistic</th>
<th>pNG6 (no SAR)</th>
<th>pNG611 (P1-SAR)</th>
<th>pNG622 (β-globin SAR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>56</td>
<td>54</td>
<td>57</td>
</tr>
<tr>
<td>Mean</td>
<td>80</td>
<td>39</td>
<td>112</td>
</tr>
<tr>
<td>Variance</td>
<td>11,369</td>
<td>736</td>
<td>10,132</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>139.9</td>
<td>68.9</td>
<td>90.7</td>
</tr>
</tbody>
</table>

a The SAR fragment flanking the gusA gene is in parentheses.
b The actual number of calli analyzed is 60, but calli without detectable GUS activities are omitted.
c The mean is given in units of enzyme per milligram of total protein.

The series are named according to the T-DNA construct they contain.
protoplast preparation would stabilize the nuclear scaffolds. Although we cannot exclude the possibility that some other factor during nuclei isolation stabilizes the internal network, our results could indicate that scaffold extraction from tobacco nuclei does not need a stabilization step.

The extracted scaffolds are composed of a mixture of some abundant and several minor polypeptides of which the majority have lower molecular weights than those of animals and yeast (Cardenas et al., 1990; Belgrader et al., 1991). Compared with the nuclear proteins, a few of the polypeptides are enriched in the scaffolds, whereas most of the histones and some other proteins have been released during extraction. In animals and yeast, several proteins copurifying with the scaffolds have been identified and some of them have specific affinity for scaffold attachment regions (Adachi et al., 1989; Gasser et al., 1989; Hofmann et al., 1989; von Kries et al., 1991). It remains to be shown which of the tobacco proteins are able to interact with the SARs, but the extraction of pure scaffolds should allow purification and identification of SAR binding proteins.

The tobacco scaffolds reproducibly bound the same genomic fragments, whereas others remained almost entirely in the supernatant together with the vector fragments. The effectiveness of binding varied with different scaffold preparations and, therefore, seems to be related more to the purity or integrity of the scaffolds rather than to the binding affinity of the fragments. Of the detected SARs in the 17.1-kb plant DNA fragment, all but one are lying in untranscribed DNA sequences. This is in agreement with data known about other SARs (Gasser et al., 1989; Surdej et al., 1990; Hall et al., 1991). The only exception is the 1.1-kb SAR at the right end of the clone, but it is still possible that this SAR lies in an intron, as has been observed for some vertebrate genes (Cockerill and Garrard, 1986b; Kás and Chasin, 1987).

If all these SARs would be constitutively scaffold attached in vivo, then the loops formed would be much smaller than what has been estimated for the bulk chromatin (Jackson et al., 1990). It is possible that the SARs define the bases of very small loops, but more likely some of them became attached to the scaffolds during the extraction procedure. Nevertheless, they have the potential to bind and might be functional SARs in vivo (Farache et al., 1990), which are scaffold attached in a transcription-dependent way. Only in the tissue or cells where a gene is expressed would the neighboring SAR become scaffold attached, leading to stable and appropriate transcription. The fact that every gene present in the clone has a different tissue specificity could support this idea and would explain why they are all flanked by a SAR. Because no data are known about the promoter and regulatory sequences of the genes, except for the lectin gene, we cannot determine whether the SARs are mapping at or near enhancer sequences as is often the case with animal genes (Jarman and Higgs, 1988; Gasser et al., 1989). We are currently investigating the presence of SARs in well-characterized plant gene loci. This will demonstrate whether certain plant SARs cohabit with regulatory sequences.

### The 1.55-kb P1-SAR Has Structural Properties Analogous to Animal SARs

The 1.55-kb P1-SAR fragment contains a 520-bp region and a 190-bp region that have an A+T content of >70% and contain the same motifs present in animal and yeast SARs (Figure 3A). The two regions are separated by a 210-bp sequence with an A+T content of 60% that does not contain any of the mentioned motifs. Cutting the 1.55-kb fragment in two demonstrated that the 520-bp fragment can associate with the scaffold, whereas the remaining 1 kb only attaches poorly. Obviously, the 190-bp AT-rich region is not enough to allow scaffold attachment. Similarly, it has been shown by Mieke et al. (1990) that animal SAR fragments of <300 bp lose most of their binding affinity; this seems to be related to the modular nature of SARs, meaning that a minimal number of repeated motifs is required for efficient binding. As in animal systems (e.g., Jarman and Higgs, 1988), competition with E. coli DNA has little effect on P1-SAR association unless very high concentrations (more than a 1000-fold excess to the probe) are added. Therefore, it seems that specific sequence elements determine P1-SAR attachment.

More substantial data about the affinity of the P1-SAR scaffold interaction came from competition experiments with the ftz SAR from Drosophila, which can also attach to tobacco scaffolds. This result demonstrated that the ftz-SAR fragment competes with the P1-SAR fragment and displaces it until a basic level of P1-SAR attachment is reached, suggesting that both SARs can attach to the scaffolds in a similar way and mainly occupy the same binding sites. These data, together with the fact that the plant SARs attach to Drosophila scaffolds, imply that scaffold attachment is conserved between Drosophila and tobacco. On the contrary, a SAR from the human β-globin domain has only very low affinity for tobacco scaffolds, although it attaches to rat scaffolds as effectively as does the P1-SAR fragment. It has been shown that exogenously added SARs bind to the scaffolds by replacing endogenous SARs (Izaurralde et al., 1988); perhaps the mode of association of the β-globin SAR is different from that of tobacco SARs and does not allow effective competition with the endogenous SARs. Alternatively, the β-globin SAR recognizes other binding sites that are not abundant in tobacco scaffolds. At any rate, these results show that evolutionary conservation of scaffold attachment does not include all SARs in every system, as has also been shown for some animal SARs (Amati and Gasser, 1990).

### The Fragment Containing the P1-SAR Is Able to Normalize Reporter Gene Expression in Independent Tobacco Transformants

Flanking a gusA reporter gene by the P1-SAR fragment results in a 60% decrease of variation in GUS activity among gusA-expressing calli compared with a non-SAR construct. Between
50 and 60 independently transformed calli of each series were tested for GUS activity, permitting justified statistical analysis. When working with animal organisms, statistical analysis is usually a problem because too few stable transformants can be obtained.

A frequency distribution of the GUS activity in the different series (Figure 6) shows that calli containing the P1-SAR construct are underrepresented in the lowest class and that none expresses the gusA gene to levels higher than 100 units of enzyme per milligram. This result leads to a distribution pattern in which most transformants are normally dispersed around the mean. In contrast, the calli of the control series are scattered over all classes with a high percentage (~40%) falling in the lowest class, a distribution pattern that is frequently obtained when analyzing a population of transgenics (Peach and Velten, 1991; P. Breyne, G. Gheysen, A. Jacobs, M. Van Montagu, and A. Depicker, manuscript in preparation). Because the selectable marker is linked to the reporter gene in our constructs, the following limitations have to be considered. The P1-SAR fragment might act by modulating the chromatin structure and thereby affect the number of active T-DNAs. However, this could not be determined because only transformants with an active T-DNA insert were obtained and analyzed. On the other hand, the activity of the P1-SAR fragment might be influenced by the chromatin structure. An active T-DNA conformation might thus facilitate or even induce the P1-SAR action.

Nevertheless, the fact that the presence of the P1-SAR fragment results in less intertransformant variability might indicate that specific sequences within this region can insulate the reporter gene from expression-influencing effects exerted by the host chromatin. Although we cannot exclude the possibility that a random DNA fragment with an A+T content similar to that of P1-SAR would have the same effect, this is most probably not the case because it has been shown previously that a high A+T content is insufficient to permit scaffold attachment or to affect gene expression (Mielke et al., 1990). This assumption is also suggested by the fact that the β-globin SAR has no potential to reduce the overall variance, which might be related directly to the limited affinity of the β-globin SAR for tobacco scaffolds; alternatively, this SAR does not contain sequences that have the capacity for insulating a reporter gene from expression-influencing effects.

Boundary elements that do insulate transgenes from such effects have already been identified in animals (reviewed by Eissenberg and Elgin, 1991) and might serve as borders that separate independent functional chromatin domains in vivo. Although it is not clear yet how they function, there seem to be different classes of boundaries of which one type appears to comap with SARs (Bonifer et al., 1991). It is possible, therefore, that the P1-SAR or sequences close to it also function as a domain boundary and can insulate the reporter gene from both suppressing and elevating effects of the surrounding chromatin, resulting in transgene expression normalizing at a level equivalent to endogenous gene activities.

METHODS

Preparation of Nuclei

Nuclei were prepared as described by Walling et al. (1988) with some modifications. The major difference is that nuclei were isolated out of protoplasts prepared from tobacco (Nicotiana tabacum cv SRI) leaves as described by De Block et al. (1984). A normal preparation started from 10^6 protoplasts concentrated in 2 mL. They were lysed in 40 mL of Honda buffer (2.5% Ficoll, 5% dextran-T40, 25 mM Tris, pH 8.5, 5 mM MgCl2, 0.5% Triton X-100, 0.44 M sucrose) supplemented with 2.5 mM DTT (instead of β-mercaptoethanol), 1 mM PMSF, 10 µg/mL leupeptin, and 2 mM spermine. After lysis (30 min on ice), the nuclei were pelleted by centrifugation at 3000g for 5 min and washed twice with the same buffer. The nuclei were resuspended in 5 mL of the lysis buffer without spermine and homogenized with two gentle strokes in a Dounce homogenizer. The nuclei were then layered on two 4-step Percoll gradients (each layer being 2 mL) and centrifuged for 30 min at 4000g in a swinging bucket rotor. The fraction containing the nuclei was recovered, washed twice, finally resuspended in 1 mL of 50 mM Tris, pH 8.5, 5 mM MgCl2, 50% glycerol, and stored at -20°C.

When starting from 10^7 protoplasts, three A260 units of pure nuclei were obtained. The amount of nuclei was determined by diluting 0.1% of the nuclei 50-fold in 1% SDS followed by phenol and chloroform extraction of the DNA, precipitation, and resuspension in water. The DNA concentration in the sample is determined by measuring the absorption at 260 nm. One A260 unit of nuclei is defined as the amount that corresponds to 50 µg of DNA.

Rat liver nuclei were prepared as described in Izaurralde et al. (1988).

Extraction of Scaffolds

The extraction of scaffolds was basically done as described by Mirikovitch et al. (1984). An amount of nuclei corresponding to three A260 units was washed three times with a buffer containing 375 mM Tris, pH 7.5, 200 mM KCl, 0.5 mM spermine, 1.25 mM spermidine, 0.1% digitonin, 1 mM PMSF, and 10 µg/mL leupeptin. The nuclei were resuspended in 200 µL of the same buffer, and 5 mL of extraction buffer (20 mM Heps, pH 7.5, 0.1 M LiAc, 1 mM EDTA, 20 mM lithium dodiumacetylamide [LiSA], and 0.1% digitonin) was added at room temperature. The tobacco nuclei were not stabilized prior to extraction, unless stated otherwise. Rat liver nuclei were stabilized at 42°C for 20 min. After extraction for 5 min at room temperature, the nuclear suspension was centrifuged for 20 min at 18000g at 4°C. The pelleted nuclear scaffolds were washed five times with an excess of digestion buffer (20 mM Tris, pH 7.5, 7 mM MgCl2, 100 mM KCl, 2.5 mM DTT, 100 µg/mL BSA, 0.1% digitonin, 1 mM PMSF, and 10 µg/mL leupeptin). After the last centrifugation (10 min at 6000g), 1 mL of digestion buffer was added to the scaffolds together with 300 units of two different restriction enzymes. After digestion at 37°C for 3 hr, 10 aliquots were made and stored frozen at -20°C. Before binding, every aliquot was diluted twice in digestion buffer and incubated with 10 units of two other enzymes for 1 hr. EDTA was then added to a final concentration of 25 mM.

Drosophila nuclei and scaffolds were prepared as described by Mirikovitch et al. (1984).

In Vitro Binding of DNA to the Scaffolds

Plasmids were cut with appropriate restriction enzymes and labeled by filling in the sticky ends (Maniatis et al., 1982). Fifty to 100 ng of
labeled plasmid was incubated with 0.3 A_{260} units of nuclear scaffolds in digestion buffer with EDTA at 37°C. After 2 to 3 hr, the scaffolds were centrifuged for 10 min at 5000g, the supernatant was kept apart, and the pellet was resuspended in the same volume of digestion buffer as present in the supernatant. DNA was recovered from both the pellet and the supernatant fraction by treatment with proteinase K in the presence of 1% SDS for 1 hr at 65°C and subsequent phenol extraction (Manniatis et al., 1982). Unless stated otherwise, samples with equal amounts of radioactive counts from pellet and supernatant were loaded on 1% agarose gels and after separation of the fragments, the gels were dried and exposed on x-ray films.

Construction of Plasmids and Transformation of Tobacco Cells

The different SAR-containing fragments were first cloned in the plasmid pNGUS. This pGUS1-derived vector (Peleman et al., 1989) contains a chimeric β-glucuronidase (gusA) gene (Jefferson et al., 1988) under control of the promoter of the nopalin synthase (nos) gene (Depicker et al., 1982) and the 3' end of the octopine synthase (ocs) gene (De Greve et al., 1982). The SAR fragments were first cloned 3' from the gusA gene in the Smal site. Unless stated otherwise, samples with equal amount of GUS enzyme present. GUS activities are given in units of enzyme per milligram of total protein (Jefferson, 1987).

Enzymatic Assays for GUS Activity

GUS activity was determined using a spectrophotometric assay described by Jefferson et al. (1987). The assays were automatized using a computer-directed microtiter plate reader (340-ATTC; SLT Labinstruments, Salzburg, Austria). The detailed protocol will be described elsewhere (P. Beven, M. De Loose, G. Gheyssen, M. Van Montagu, and A. Depicker, manuscript in preparation). Briefly, reaction mixtures were made directly in the wells of a microtiter plate and incubated at 37°C in the reader. At fixed, programmed time points, the absorption at 415 nm was measured, and at the end of the reaction, the computer program calculated the mean absorption per minute for every well and the amount of GUS enzyme present. GUS activities are given in units of enzyme per milligram of total protein (Jefferson, 1987).

Statistical Analysis

Statistical tests (Sokal and Rohlf, 1981) were performed using a package of computer programs (Applied Biostatistics Inc.). Both the Bartlett's test and the log-ANOVA determine whether the variances of two different series are homogeneous. A Mann-Whitney U-test determines whether the items of two different groups are equally distributed.

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