A plant scaffold attached region detected close to a T-DNA integration site is active in mammalian cells

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ABSTRACT

Integration of foreign genes into plant genomes by the *Agrobacterium* T-DNA transfer system has been considered to occur at random. It has been speculated that the chromosomal structure of the integration site might affect the expression pattern of the introduced genes. To gain insight into the molecular structure of T-DNA integration sites and its possible impact on gene expression, we have examined plant DNA sequences in the vicinity of T-DNA borders. Analysis of a transgenic petunia plant containing a chloramphenicol acetyltransferase (CAT) gene regulated by the hemoglobin promoter (PAR) from *Parasponia andersonii* revealed a scaffold attachment region (SAR) close to one T-DNA end. In addition to having strong binding affinities for both animal and plant nuclear scaffolds this petunia SAR element is as active in mammalian cells as the authentic elements from mammalian sources.

INTRODUCTION

So far, the expression level of genes introduced into the plant genome is not predictable and can vary substantially between individual plants transformed with the same gene construct (1–4), in some cases to the extent of loss of organ specificity (5). Gene expression has been positively as well as negatively correlated with gene copy numbers (2, 6, 7). This variability of expression has been referred to as 'position effect'. It may partly be due to endogenous regulatory elements like promoters or enhancers close to the integration site and to the chromosomal structure/transcriptional state of the integration site. Other factors involved in the variability of foreign gene expression are DNA methylation of regulatory sequences (8–10) and the availability of *trans*-acting factors.

The higher order chromatin structure of the eukaryotic genome is thought to play an important role in replication and transcription processes. According to current models, chromatin is organized in chromosomal loop domains, the basal parts of which are attached to a proteinaceous scaffold, also referred to as the nuclear

matrix (11, 12). Accordingly, the responsible DNA sequences have been called 'scaffold-attached regions' (SARs) or matrix-attached regions (MARs). These S/MAR elements insulate chromatin domains from neighbouring loops with respect to their chromatin structure and torsional state. They are thought to represent the tools permitting a differential regulation of transcriptional processes (13, 14). Chromatin loops encompass between five and more than one hundred kilobases. Their attachment to the nuclear matrix is mediated by component proteins which *in vitro* have a high and specific affinity for SAR sequences (15).

There are several points of evidence that T-DNA transferred into plant cells via *Agrobacterium tumefaciens* is preferentially integrated into transcriptionally active regions of the plant genome. A high frequency of reporter gene activity is observed after integration of promoterless reporter genes which is obviously due to gene fusions to endogenous promoters (16–18). Moreover, it has been shown for crown gall tumor lines (19) as well as for transgenic tobacco plants (20) that genes which are transferred by the *Agrobacterium* Ti plasmid are integrated in DNase I-sensitive domains and hence are associated with an 'open' chromatin conformation.

The preferential T-DNA insertion into potentially transcribed genome regions may be mediated by the T-DNA integration mechanism which seems to require DNA replication and repair (21). Alternatively, the 'open' and thus potentially active chromatin conformation typically found at the integration site may be a consequence of the *in vitro* selection process, i.e. only transformants with T-DNA being integrated into permissive chromatin domains might be recovered during selection for the expression of the transferred antibiotic resistance markers.

The aim of our current efforts is to obtain more information on the contribution of plant DNA sequences surrounding T-DNA integration sites to local chromatin structures and to transcriptional activity. As a first step we here provide a detailed analysis of the molecular structure of a T-DNA integration site and its possible impact on gene expression.

Petunia plants were transformed with a chloramphenicol acetyltransferase (CAT) gene driven by the hemoglobin promoter

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(PAR) from *Parasponia andersonii*. This promoter has been shown to confer root specific expression in transgenic tobacco (22), however, leakiness of organ specificity has been observed in a number of transformants of tobacco (5, 22) and petunia (Dietz and Landsmann, unpublished). The leakiness in organ specificity of the PAR promoter in some transformants may be ascribed to influences of the integration locus. A transgenic petunia transformant with strong albeit not root specific expression was selected for molecular analysis of the integration site. We demonstrate that a T-DNA integration has occurred close to a scaffold attachment region (SAR).

MATERIALS AND METHODS

Strains and plasmids

The binary vector pPAR4925 was constructed by J. Landsmann (22). *Agrobacterium tumefaciens* strain LBA4404, which carries the helper Ti plasmid pAL4404 (23), was used for transformation of *Petunia hybrida* var. RL01. Cloning of plant DNA was done in lambda ZAPII (Stratagene) using *Escherichia coli* strain SURE (Stratagene) as a host.

A 2.2 kb EcoRI fragment (SAR₂₂₀₀) and its 0.83 kb subfragment (SAR₈₀₀), derived from the human interferon- β upstream SAR element, were each cloned into the polylinker of plasmid pTZ18R (24, 25). Similar elements flanking the immunoglobulin heavy chain enhancer have been described as 'MARs' by Cockerill and Garrard (26). These elements, donated by T. Kohwi-Shigematsu (San Diego) were cloned into plasmid pUC19 and incorporated in the studies covered by Figure 4 (fragments 'IgH_L' and 'IgH_R'). The oligonucleotide multimer (heptamer) containing the nucleation site of unwinding in the MAR of this IgH enhancer (27) was subcloned into the BamHI/SalI site of the polylinker of plasmid pTZ18R (fragment 'heptamer' in Figure 4).

SAR-containing transfection plasmids are based on pLu (28). Constructs Lu, ELu and LuP have been described previously (28). LuI and ELuI were cloned by inserting a 3 kb BglII-fragment containing 'I' (downstream of the human β -interferon gene) into the NruI-site of Lu and ELu, resp. LuPS and ELuPS were cloned in the same manner using the 1.65 kb HindIII-XbaI fragment from petunia clone Nos1.1 (see Figure 2).

Plant transformation

Transformation of petunia was done by the leaf disc method (29). The recombinant binary vector pPAR4925 (22) was mobilized from *E. coli* to *Agrobacterium tumefaciens* through triparental mating with the conjugative helper plasmid pRK2013 in *E. coli* HB101 (30). Shoots regenerated on MS9 Agar medium (Flow Lab.) supplemented with 20 g/l sucrose, 0.1 mg/l α -naphthalene-acetic acid, 1.0 mg/l 6-benzylamino-purine and 250 mg/l Claforan (Hoechst). Transformants were selected on 100 mg/l kanamycin sulphate.

Biochemical analysis of transgenic plants

Extracts of root or leaf tissue were prepared as described by Delauney *et al.* (31). After determining protein concentrations by the Bradford colorimetric assay (BioRad), 30 μ g of total protein were used for CAT enzyme assays using 0.025 μ Ci of ¹⁴C chloramphenicol (53 mCi/mmol, Amersham) and 1 mM AcetylCoA at 37°C for 30 min. Reaction products were extracted with ethyl acetate and subjected to chromatography on a silica

gel thin-layer plate (Merck) with chloroform/methanol (95:5) as eluent. TLC plates were exposed to Agfa Curix RP1 film for at least 20 hr.

DNA isolation and analysis

Plant genomic DNA was prepared from leaves as described by Dellaporta *et al.* (32). For Southern analysis, 10 μ g of restriction enzyme digested plant DNA was separated on a 0.8 % agarose gel and transferred to Hybond-N (Amersham) nylon membrane by alkaline transfer (33). Membrane blots were hybridized according to the manufacturer's protocol using DNA fragments radiolabelled with ³²P by the random primer method (34). Plasmid DNA was isolated according to Birnboim and Doly (35).

Cloning of plant DNA

Nuclear DNA was digested with SacI and fractionated on a sucrose gradient. DNA fractions in the size range of 2.5–8 kilobases were ligated to Lambda ZAPII which had been cut with SacI and treated with alkaline phosphatase. Ligated DNA was packaged with Gigapack II Gold (Stratagene), yielding $>2 \times 10^6$ recombinant phages per μ g DNA. The library was plated on 24 $\times$ 24 cm square Nunc plates, transferred to nylon membranes (Amersham) and hybridized to radiolabelled DNA fragments. Positive phage clones were isolated and subjected to an *in vivo* excision of the corresponding phagemid (36).

DNA sequencing

Nucleotide sequences at the integration site were determined by the dideoxynucleotide chain termination method (37) using a Sequenase kit (United States Biochemical). The 1.65 kb HindIII-XbaI fragment of Nos1.1 was subcloned in pGEM3Zf(+) (Promega), thereby creating plasmid pGN1.5 which was subjected to digestion with Exonuclease III prior to sequence analysis.

Isolation of nuclei

The procedures for culturing mouse-L cells, and the isolation of nuclei were as described (24, 38). Two plates of 150 cm² (5×10^7 cells) were washed twice with isolation buffer (3.75 mM Tris-HCl pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM K-EDTA, 20 mM KCl, and 1% thioglycol, freshly adjusted to 0.2 mM PMSF). They were then scraped off in 15 ml of isolation buffer containing 0.1% digitonin. Nuclei were set free by 15 strokes in a tightly-fitting Dounce homogenizer. After centrifugation (900 g, 5 min., 4°C), they were resuspended once in the same medium and pelleted as before.

The procedure for the isolation of plant nuclei was adopted from Watson and Thompson (39) and from Slatter *et al.* (40). Leaves from 10-day-old pea shoots were treated with ice-cold ether, washed several times with extraction buffer (1 M hexylene glycol, 10 mM Pipes-KOH, pH 7.0, 10 mM MgCl₂, 5 mM mercaptoethanol) and homogenized with a blender for 10 sec. The homogenate was filtered through a 500 μ m nylon mesh, and Triton X-100 was added to a final concentration of 0.5%. After subsequent filtrations through 100-, 55-, 20 μ m nylon mesh the nuclei were pelleted. The pellet was suspended in extraction buffer and layered onto a 40%, 60%, 80% (v/v) Percoll step gradient on top of a 7 ml cushion of 2 M sucrose in gradient buffer (0.5 M hexylene glycol, 10 mM Pipes-KOH, pH 7.0, 10 mM MgCl₂, 5 mM mercaptoethanol). After centrifugation

at 3500 g for 30 min, the nuclei banding in the sucrose pad were washed twice with gradient buffer, suspended in nuclear isolation buffer (1 M hexylene glycol, 1% thioglycol, 20 mM KCl, 20 mM Hepes, pH 7.4, 0.5 mM EDTA, 0.05 mM spermine, 0.125 mM spermidine, 0.2 mM PMSF, aprotinin at 5 µg/ml, 10 µM E-64), adjusted to 50% glycerol and stored at -80°C.

Nuclear scaffolds

Procedures were essentially as described before (25) and were found applicable for nuclei from both plant and mammalian sources. Nuclei from 2×10^7 mouse-L cells or from 14 g of pea (*Pisum sativum*) leaves were subjected to the lithium 3,5-diiodosalicylate (LIS)-extraction procedure of Mirkovitch *et al.* (15). To obtain nuclear halos they were suspended in 100 µl of 5 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 1% thioglycol, 0.1% digitonin, 0.2 mM PMSF. After the stabilization step (incubation at 42°C for 30 min), the nuclei were resuspended in 2 ml of LIS-dilution buffer (20 mM HEPES-NaOH, pH 7.4, 0.1 M lithium acetate, 1 mM EDTA, and 0.1% digitonin) by use of a loosely fitting Dounce homogenizer. Extraction of non-scaffold proteins was then achieved by adding 2 ml of 50 mM LIS dissolved in the same buffer (final LIS-concentration: 25 mM) and by carefully homogenizing the mixture by two strokes. After pelleting the extract (2400 g, 5 min., 4°C) the resulting nuclear halos were washed 4 times in 20 ml of sterile digestion buffer (20 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 20 mM KCl, 70 mM NaCl, 10 mM MgCl, 0.2 mM PMSF). To obtain the scaffolds halos were degraded with an appropriate set of restriction endonucleases, depending on the type of experiment and the type of DNA used (see the figure legends for details); digested DNA remained within the mixture as part of the competitor DNA that is required to induce specific binding (see Ref. 24 and below).

Binding experiments

Scaffold preparations were adjusted to 700 µl with digestion buffer containing *E. coli* genomic DNA at a final concentration of 0.66 mg/ml (corresponding to a $> 10,000$ fold molar excess over the labelled DNA fragments). For standard reassociation assays they were partitioned into aliquots (5×10^6 cell equivalents of nuclear scaffolds each) and provided with restriction fragments that had been end-labelled by Klenow-polymerase using α - 32 P-dATP. All samples were agitated at 37°C overnight. After separation into pellet (P) and supernatant (S) fractions by centrifugation (4°C, 14000 g, 45 min), samples were subjected to DNA purification (25). Equal counts of P and S dissolved in 100 µl Tris-EDTA buffer were separated on 1% agarose gels ('equal counts' approach, cf. 41). After electrophoresis, the gels were dried onto Whatman 3MM paper using a heated gel dryer or the DNA was blotted onto a Nylon screen (BioRad Zeta probe), and autoradiographed overnight by exposure to Agfa Curix RP1 or Kodak X-Omat AR film.

Gene transfer to rodent cells

Mouse-L cells were grown as described (38). For stable expression experiments, the transfection protocol has been optimized to yield low copy numbers (28, 42). This has been achieved by omitting carrier DNA and by transferring linearized DNA (as opposed to supercoiled DNA) which reduced average copy numbers about five-fold. All data from stable-expression

experiments were referenced to the number of integrated copies which was determined as described before (42).

For the transient expression experiments in Figure 5 (insert), 10^5 cells were transfected in a six-well plate using a calcium(2+) phosphate coprecipitate from 2.5 µg of the respective pLu derivative together with 2.5 µg of pRSV/SEAP and no carrier DNA. pRSV/SEAP, which is based on a secretory alkaline phosphatase indicator gene (43), served as an internal reference to correct for variations in the transfection efficiency.

Luciferase tests

Extracts were directly prepared from a defined number of cells. Usually, 10^5 – 10^6 cells were lysed in 300–1000 µL of extraction buffer (0.1 M KH_2PO_4 , pH 7.8, 1 mM dithiothreitol). The bioluminescence of 10–50 µL was recorded in a Berthold Biolumat model LB 9500c by integrating the output over the first 10 seconds and correcting this value for the number of cells (white bars in Fig. 5) and, in addition for the number of intact copies in the recipient cells (dark bars in Fig. 5). Control experiments established that bioluminescence data reflect transcriptional levels (44).

RESULTS

Cloning of T-DNA borders

Petunia plants transformed with pPAR4925 and selected for kanamycin resistance were grown under sterile conditions. As expected, all of the kanamycin resistant transformants displayed

Figure 1. Restriction map of phagemid clone Nos1.1. A: outline of pPAR4925 T-DNA regions and probe fragments used in screening. B: T-DNA and adjacent plant DNA as contained in Nos1.1. S, SacI; RB, right border; LB, left border.

strong CAT enzyme activity in roots. Some plants, however, at times expressed the CAT reporter gene in leaves as well. One of these transformants (P13) was subjected to selfing and backcrossing. Root and leaf expression of the CAT gene was observed in all of the kanamycin resistant progeny tested. The extent of leaf expression in the original transformant P13 and in the progeny was dependent on the physiological state and on the position of the leaf, often being in the same range as the expression in roots. P13 was analyzed at the molecular level to determine the copy number of integrated PARCAT sequences. Blot hybridization of P13 DNA indicated the presence of 3 tightly coupled gene copies (data not shown). This is in accord with the segregation ratio.

In order to determine more precisely the structure of the integrated T-DNA in transformant P13, we constructed a genomic lambda phage library. The library was screened with the probes nos3' or PARCAT (Figure 1A) until recombinant phagemid clones containing right border (RB-) or left border (LB-) fragments, respectively, were obtained. The right border clone, designated Nos1.1 (Figure 1B), recognizes a single BamHI fragment of 3.8 kb when hybridized with genomic DNA from untransformed petunia (data not shown). Recognition of single plant DNA fragments was verified using different restriction enzymes and two Nos1.1 subfragments. Because of the presence of a unique (single copy) plant DNA sequence, Nos1.1 was subjected to a more detailed sequence analysis, in order to obtain information on the structure of a potentially active DNA region.

The right T-DNA border is located close to an A+T-rich region

The nucleotide sequence of the right border integration site on Nos1.1 was determined using synthetic sequencing primers from the T-DNA right border region of the nopaline Ti plasmid pTiT37 (45). The T-DNA boundary occurs within the 25 bp imperfect repeat defining the right end of the T-DNA. The plant DNA immediately adjacent is extremely A+T-rich (80% within the first 21 nucleotides), as has been reported for other T-DNA integration sites (46, 47). No open reading frame was found in

Figure 2. Subfragments of Nos1.1, identified by their hybridization characteristics. The upper map shows the phagemid, linearized at the multicloning site of pBluescript. The bar below represents the subfragment probe used (the open part depicts the 1.8 kb region of weak hybridization while the closed part represents fragments which hybridized at a normal strength). The lower map shows the 1.65 kb XbaI-HindIII petunia DNA fragment which was subcloned to create plasmid pGN1.5. B, BstEII; C, ClaI; H, HindIII; Hp, HpaI; S, SacI; Sc, ScaI; Sp, SpeI; X, XbaI; RB, right border.

the plant DNA at the right T-DNA border integration site of Nos1.1.

Upon hybridization of Nos1.1 subfragments with untransformed genomic DNA from petunia we identified a 1.8 kb BstEII-HindIII fragment (Figure 2) showing base pairing to plant genomic DNA and to itself only at nonstringent conditions (hybridization temperature 59°C as compared to 68°C used for stringent conditions). For further investigation of this presumably

Figure 3. Scaffold binding potential of subfragments from plasmid pGN1.5. A, A standard reassociation using mouse Ltk⁻ derived scaffolds (digested with BamHI, EcoRI and HindIII) was performed with different radiolabelled fragments of pGN1.5 using 5 fmols of each digest per reassociation (5×10^6 cell equivalents). The 3.2 kb pGEM vector fragment serves as a negative control. As a standard, a radiolabelled EcoRI/HindIII digest of pCL (25) comprising the 800 bp human interferon- β upstream SAR (SAR₈₀₀) and the non-binding 2.9 kb pTZ vector fragment was included; binding of SAR₈₀₀ is 95–100% throughout this gel and serves for quantification. For each track 2000 cpm were applied, and these were aliquots of the following total activities: 1: P, 44200; S, 47600; 2: P, 46100; S, 58440; 3: P, 38570; S, 69480; 4: P, 44220; S, 97570 cpm. Lanes r_m contain, as a reference, the different restriction fragments of pGN1.5 that were used: 1, XbaI/HindIII; 2, XbaI/HindIII/HpaI; 3, XbaI/HindIII/SpeI; 4, XbaI/HindIII/SpeI/AflIII. Lane r_s marks the binding/non-binding standards from pCL; P, pellet-, S, supernatant fraction. The fragments generated after digestion with the respective restriction enzymes are indicated. B, Binding strength of petunia SAR and subfragments thereof for Ltk⁻ nuclear matrices. The autoradiograph from Fig. 3A was scanned and binding of the respective band was expressed as % binding, i.e. $\text{cpm}_p \times 100 / (\text{cpm}_p + \text{cpm}_s)$ using SAR₈₀₀ as a standard. Symbols adjacent to the bars mark ATATTT-motifs (vertical arrowheads), inverted repeats (horizontal arrowheads facing each other), (dA)_n-tracts (numbers mark n-values), segments conforming to the AAA(N)₇₋₁₀AAA bending criterion (dashes).

A+T-rich region, a 1.65 kb XbaI–HindIII fragment (indicated in Figure 2) was subcloned and subjected to DNA sequence analysis. The overall A+T content of this region is 70%, but is higher distal to the integration site at positions 1390–1664 (79%) and 1060–1664 (75% A+T-rich region).

Scanning of the 1664 bp sequence obtained from the weakly hybridizing region revealed several motifs common to scaffold-attached regions. The T-box [10 bp consensus TTA/TTT/

Figure 4. Comparison of binding affinities of mouse and pea nuclear scaffolds (each digested using BamHI and HindIII) for different SAR elements. Nuclear matrices of mouse Ltk⁺ cells (left) and pea leaves (right) were both incubated with the same set of radioactively labelled restriction fragments. For application onto an agarose gel the equal counts approach was followed. T, mixture used for incubation with scaffolds, P, pellet-, S, supernatant fraction, Petun-SAR, 1.65 kb XbaI–HindIII petunia SAR fragment.

ATTT/ATT] (48) is present 4 times in the 604 bp stretch most distal to the integration site. The sequence ATATTT, occurring 7 times in the 1664 bp fragment, has been shown to be overrepresented in SARs from animals (25, 26) and from plants (25, 40) and conforms to the center of the *Drosophila* topoisomerase II consensus sequence. Other elements within the 1664 bp A+T-rich region which are common to several SARs (40, 49, 50) are two 10 out of 11 bp matches of a yeast ARS sequence. One of these is in close proximity to three T-boxes and to several direct and inverted repeats differing in length from 10–14 bp. Direct and inverted repeats have previously been identified in scaffold associated regions (SARs or MARs) from various organisms (40, 48, 51, 52). A 7-bp dyad symmetry is located around position 1620.

***In vitro* binding of cloned plant DNA fragments to nuclear scaffolds**

Our assumption that the sequenced plant DNA close to the right T-DNA border represents a SAR element was verified by subjecting the cloned 1.65 kb XbaI–HindIII fragment and several of its subfragments to a standard *in vitro* binding assay, based on a scaffold preparation from murine cells (25). Figure 3A shows an experiment which correlates the SAR activity of these DNAs with that of an authentic 800 bp SAR subfragment derived from the human Interferon- β domain borders (fragment IV, cf. 25). Equal counts of the pellet and supernatant fractions were applied to the P and S lanes of an agarose gel. Under these conditions it becomes evident that the affinity of the XbaI–HindIII, HpaI–HindIII, XbaI–SpeI and SpeI–HindIII fragments is at least as high as that of the control (SAR₈₀₀) while the shorter fragments are below this threshold. This is represented in Figure 3B schematically, following the convention introduced

Figure 5. Effect of various SAR elements and combinations thereof on luciferase expression in stably transfected cells. Transcriptional enhancement is shown as relative expression referring to Lu (=1), the plasmid which all other constructs are based on. Light bars: immediate expression levels for a representative set of transfection experiments. Solid bars: expression corrected for the number of integrated copies (averages from four independent transfections, lines representing standard deviations). Insert: Luciferase expression from the same constructs in transiently transfected cells. Lu, firefly luciferase; E, SAR upstream of the human interferon- β gene, I, SAR downstream of the human interferon- β gene, P, SAR upstream of the potato ST-LS1 gene (67), PS, petunia SAR.

in Ref. 25. Since the total radioactive counts of fragments in the pellet and in the supernatant differed only up to twofold (see legend to Figure 3A), an 'equal fractions' approach would have yielded the same classification.

We ascribe the high (> 80%) binding potential of the equally sized XbaI–SpeI and SpeI–HindIII fragments to the presence of adequately spaced ATATTT motifs (25). A close inspection of Figure 3A, however, reveals a somewhat stronger association of the more distal SpeI–HindIII fragment which harbours the bulk of motifs implied in scaffold binding. The 180 bp SpeI fragment is released from the scaffold, as had to be expected regarding its small size (cf. 25).

If the isolated sequence represents an authentic SAR element in plants, it should be possible to demonstrate a similar behaviour using a plant scaffold. We have therefore prepared scaffolds from pea leaves following the protocol developed for murine cell nuclei. Binding assays comparing the relative affinity of a variety of sequences for mouse and pea scaffolds are shown in Figure 4. Binding affinities with pea and with mouse-L cell derived scaffolds are comparable for most of the fragments used for calibration (Petun–SAR = SAR₂₂₀₀ > IgH_r > IgH_i > Heptamer). An apparent difference arises for the 800 bp SAR fragment from the interferon- β gene (SAR₈₀₀) which under the conditions of the experiment is partially released by the pea scaffold whereas it is still bound by the murine counterpart.

The petunia SAR enhances gene expression in stably transfected mouse cells

SAR elements cause an enhanced transcription of a linked reporter gene when the construct is stably integrated into the genome of a mouse recipient cell. The same behaviour is also found for the petunia element (Figure 5). When using a single SAR either upstream or downstream of the reporter gene, the increase in expression is 2- to 3-fold (compare constructs LuP, LuI and LuPS carrying the upstream SAR from the tobacco ST-LS1 gene, the downstream SAR from the human IFN- β gene or the petunia element at a downstream position and construct ELu bearing the upstream SAR from human IFN- β at an upstream position). A more pronounced enhancement effect (up to 16 fold) is seen in cases where the gene is flanked by two SARs forming a mini domain. A comparison of constructs ELuP, ELuI and ELuPS shows that these elements operate in an additive (ELuP) or even synergistic way (ELuI and ELuPS). No comparable effect can be seen in a transient assay (Figure 5, insert) clearly ruling out that the elevated levels of expression are due to the presence of classical enhancer elements.

DISCUSSION

We have introduced a reporter gene, CAT, driven by the plant hemoglobin PAR promoter, into petunia via T-DNA mediated transfer. A petunia plant with an unexpected expression pattern was selected for further analysis in order to characterize the flanking chromatin structures possibly contributing to the failure of the hemoglobin promoter to confer an exclusively root specific expression. For the respective plant, a SAR element was detected close to the site of integration. The characterization of this element is the subject of the present study which is part of a more extended program on molecular aspects of tissue specific expression.

SARs have first been identified in several animal systems and in yeast (24, 53–55). Short SAR sequences are a few hundred base pairs in size and are typically found in close association with

known enhancer elements as demonstrated for the immunoglobulin heavy and kappa chain enhancers (26, 56) and several developmentally regulated *Drosophila* genes (48). Other SAR elements cover several kilobases, they mark regions of decreasing accessibility to DNase I and are therefore candidates for the borders of chromatin domains (13, 24, 57–59). Plant SARs have been identified downstream of the pea plastocyanin gene (40), downstream of the soybean heat shock gene (60) and upstream as well as downstream of the light-inducible potato ST-LS1 gene (Bode, unpublished).

A number of features has been ascribed to SAR elements which discriminate them from other *cis*-acting sequences such as the classical enhancers and locus-control-regions (LCR). (i) it has been demonstrated by several authors that SAR functions are conserved across species boundaries (reviewed in 58, 59). One example concerns a 1.3 kb SAR fragment of a tobacco gene which behaves like mammalian SAR elements by *in vitro* and *in vivo* criteria (27). (ii) SARs elevate transcriptional levels of linked genes after, but not prior to integration of the construct into the genome of the host cell. This clearly emphasizes the importance of the final chromatin structure for the action of these elements. (iii) SAR elements forming an efficient mini domain by flanking a gene at either end cooperate, frequently in a synergistic manner (42, 61).

In the present case we show that the petunia sequences close to the site of T-DNA integration meet all these criteria. The 1.65 kb XbaI–HindIII fragment harbours many of the common SAR motifs (concentrated in its SpeI–HindIII part most distal to the integration site, see Figure 3B), and its subfragments associate in a predictable way with a heterologous scaffold as it is routinely derived from cultured mouse cells (Figure 3). We also demonstrate that a plant nuclear scaffold isolated from pea leaves exhibits high binding affinity for the 1.65 kb petunia SAR as well as for an animal SAR element of comparable length (SAR₂₂₀₀ from the human interferon- β upstream domain border). Most of the other fragments also associate in a manner comparable to that obtained with murine scaffold preparations. The relatively weak association of the human SAR₈₀₀ fragment may only be an apparent exception caused by a lower density of binding sites in the plant relative to the mammalian scaffold. Under the experimental conditions, lanes 4 and 5 (Figure 4) clearly prove a higher binding strength for the XbaI–HindIII fragment (Petun–SAR) compared to SAR₈₀₀. This situation resembles a murine scaffold which has been overloaded with the same fragment mixture (data not shown).

The 1.65 kb petunia fragment can be used interchangeably with the mammalian fragments applied in previous assays (28, 42) to mediate a 2- to 3-fold transcriptional enhancement in stably transfected mouse cells if present on one side of the reporter gene. Moreover, the petunia SAR cooperates with the respective elements from mammalia in that the transcriptional enhancement increases considerably if the petunia element is supplemented by a second (human) SAR on the other side of the gene. Such a finding supports the minidomain concept which postulates that any two sequences qualifying as SARs by *in vitro* criteria create an independent domain when flanking a gene construct.

The generation of an artificial minidomain in the recipient cell requires two flanking SARs. If an experiment is designed to elucidate the properties of a certain SAR element, both flanks will usually consist of the same sequence. However, to provide long-term stability of gene expression it has proven advantageous to select elements of different sequence content. Thus the

combination of SARs from species as apart as plants and mammals appears to be an advantage. It is demonstrated in Figure 5 that in a stably transfected minidomain (ELuPS) the petunia SAR (PS) cooperates with an analogous human element (E). In contrast no stimulation of transcription was observed for the transient expression (Figure 5, insert). The absence of an effect prior to integration, and the activity across species boundaries are the two criteria which clearly distinguish SAR elements from classical enhancers.

Besides their effect on transcriptional levels, SARs were found to shield an integrated transgene from influences of surrounding chromatin, thereby conferring a position independent gene expression to stably integrated genes (28, 62). This property has also been demonstrated for tobacco calli transformed via *Agrobacterium tumefaciens* with a reporter gene flanked on both sides by a plant SAR (63). The fact that in this case the element did not increase expression might be ascribed to the mechanism of T-DNA integration which directs transgenes predominantly to active gene loci. SAR elements provided as part of the transgene construct should then cause no additional activation but should mainly reduce the effect of neighbouring *cis*-regulatory elements. In line with this hypothesis, a more than 20-fold increase in the expression of a reporter gene has been reported for a SAR used in stable transformation of tobacco calli by direct rather than *Agrobacterium* mediated gene transfer (64).

Whether the occurrence of SAR elements close to an integration site is as common for T-DNAs as it is in the case of animal retroviruses (65) remains to be analyzed by the characterization of more transformants. In the present case such an element was detected at the integration site of a transformant (P13) in which the normally root-specific PAR promoter was deregulated. Since the three T-DNA copies are integrated at a single locus they are expected to be equally affected by influences of the surrounding chromatin.

Deregulation of tissue specificity suggests that the *cis*-acting regulatory elements of the transgenes are not shielded from the genomic surroundings. Therefore it is considered unlikely that integration has occurred at a central position of the SAR, thereby creating a minidomain. Rather it can be assumed that integration has taken place at one of the SAR borders, in which case the expression pattern of the transgenes will be under the control of the respective host domain. It should be noted in this context that SAR elements are generally observed in close association with the regulatory elements of tissue specific rather than with housekeeping genes (66). We are currently testing transgenic constructs with two petunia SAR elements flanking a reporter gene driven by the PAR promoter, in order to demonstrate that the petunia SAR itself does not contain a tissue specific enhancer. In this case we would expect that all the transgenic plants, without exception, reflect the tissue specificity of the PAR promoter.

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REFERENCES

- Dean, C., Jones, J., Favreau, M., Dunsmuir, P. and Bedbrook, J. (1988) *Nucl. Acids Res.*, 16, 9267–9283.
- Jones, J.D.G., Gilbert, D.E., Grady, K.L. and Jorgensen, R.A. (1987) *Mol. Gen. Genet.*, 207, 478–485.
- Odell, J.T., Nagy, F. and Chua, N.-H. (1987) In Key, J. and McIntosh, L. (eds.), *Plant Gene Systems and their Biology*. Alan R. Liss, New York, Vol. 62, pp. 321–329.
- Rogers, S.G., O'Connell, K., Horsch, R.B. and Fraley, R.T. (1985) In Zaitlin, M., Day, P. and Hollaender, A. (eds.), *Biotechnology in Plant Science*. Academic Press Inc., London, pp. 219–226.
- van der Hoeven, C., Dietz, A. and Landsmann, J. *Transgenic Research*, in press.
- Gendloff, E.H., Bowen, B. and Buchholz, W.G. (1990) *Plant Mol. Biol.*, 14, 575–583.
- Shirsat, A.H., Wilford, N. and Croy, R.R.D. (1989) *Plant Sci.*, 61, 75–80.
- Linn, F., Heidmann, I., Saedler, H. and Meyer, P. (1990) *Mol. Gen. Genet.*, 222, 329–336.
- Matzke, M.A., Primig, M., Trnovsky, J. and Matzke, A.J.M. (1989) *EMBO J.*, 8, 643–649.
- Weber, H., Ziechmann, C. and Graessmann, A. (1990) *EMBO J.*, 9, 4409–4415.
- Paulson, J.R. and Laemmli, U.K. (1977) *Cell* 12, 817–828.
- Berezney, R. and Coffey, D. (1974) *Biochem. Biophys. Res. Commun.* 60, 1410–1419.
- Bonifer, C., Hecht, A., Saueressig, H., Winter, D.M. and Sippel, A.E. (1991) *J. Cell. Biochem.*, 47, 99–108.
- Goldman, M.A. (1988) *BioEssays*, 9, 50–55.
- Mirkovitch, J., Mirault, M.-E. and Laemmli, U.K. (1984) *Cell* 39, 223–232.
- Herman, L., Jacobs, A., Van Montagu, M. and Depicker, A. (1990) *Mol. Gen. Genet.* 224, 248–256.
- Kertbundit, S., De Greve, H., Deboeck, F., Van Montagu, M. and Hernalsteens, J.-P. (1991) *Proc. Natl. Acad. Sci. USA*, 88, 5212–5216.
- Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Z., Körber, H., Redei, G.P. and Schell, J. (1989) *Proc. Natl. Acad. Sci. USA*, 86, 8467–8471.
- Coates, D., Taliercio, E.W., Gelvin, S.B. (1987) *Plant Mol. Biol.*, 8, 159–168.
- Weising, K., Bohn, H. and Kahl, G. (1990) *Developmental Genetics*, 11, 233–247.
- Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Bakkeren, G., Cramer, A., Angelis, K., Redei, G.P., Schell, J., Hohn, B., Koncz, C. (1991) *EMBO J.*, 10, 697–704.
- Landsmann, J., Llewellyn, D., Dennis, E.S. and Peacock, W.J. (1988) *Mol. Gen. Genet.*, 214, 68–73.
- Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983) *Nature*, 303, 179–180.
- Bode, J. and Maass, K. (1988) *Biochemistry*, 27, 4706–4711.
- Mielke, C., Kohwi, Y., Kohwi-Shigematsu, T. and Bode, J. (1990) *Biochemistry*, 29, 7475–7485.
- Cockerill, P.N., Garrard, W.T. (1986) *Cell*, 44, 273–282.
- Bode, J., Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C. and Kohwi-Shigematsu, T. (1992) *Science*, 255, 195–197.
- Klehr, D., Maass, K. and Bode, J. (1991) *Biochemistry*, 30, 1264–1270.
- Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholtz, D., Rogers, S.G. and Fraley, R.T. (1985) *Science*, 227, 1229–1231.
- Ditta, G., Stanfield, S., Corbin, D. and Helinski, D.R. (1980) *Proc. Natl. Acad. Sci. USA*, 77, 7347–7351.
- Delauney, A.J., Tabaeizadeh, Z., Verma, D.P.S. (1988) *Proc. Natl. Acad. Sci. USA*, 85, 4300–4304.
- Dellaporta, S.L., Wood, J., Hicks, J.B. (1985) In Malmberg, R., Messing, J. and Sussex, I. (eds.), *Molecular Biology of Plants: A Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 36–37.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.*, 132, 6–13.
- Birnboim, H.C. and Doly, J. (1979) *Nucl. Acids Res.*, 7, 1513–1523.
- Short, J.M., Fernandez, J.M., Sorge, J.A. and Huse, W.D. (1988) *Nucl. Acids Res.*, 16, 7583–7600.
- Sanger, F., Nicklen, S., Coulson, S.R. (1977) *Proc. Natl. Acad. Sci. USA*, 74, 560–564.
- Bode, J., Pucher, H.J. and Maaß, K. (1986) *Eur. J. Biochem.* 158, 393–401.
- Watson, J.C. and Thompson, W.F. (1988) *Methods for Plant Molecular Biology* (A. Weissbach, H. Weissbach eds.). Academic Press, San Diego, pp. 57–75.
- Slatter, R.E., Dupree, P., Gray, J.C. (1991) *Plant Cell*, 3, 1239–1250.
- Kay, V. and Bode, J. (1994) *Biochemistry* 33, 367–374.
- Klehr, D., Schlake, T., Maass, K., and Bode, J. (1992) *Biochemistry*, 31, 3222–3229.

43. Berger, J., Hauber, J., Hauber, R., Geiger, R. and Cullen, B. R. (1988) *Gene* 66 1, 1–10.
44. Schlake, T. (1994) Dissertation, Technische Universität Braunschweig.
45. Yadav, N.S., Vanderleyden, J., Bennett, D.R., Barnes, W.M. and Chilton, M.-D. (1982) *Proc. Natl. Acad. Sci. USA*, 79, 6322–6326.
46. Gheysen, G., Van Montagu, M., Zambryski, P. (1987) *Proc. Natl. Acad. Sci. USA*, 84, 6169–6173.
47. Slightom, J.L., Jouanin, L., Leach, F., Drong, R.F. and Tepfer, D. (1985) *EMBO J.*, 4, 3069–3077.
48. Gasser, S.M., Laemmli, U.K. (1986) *Cell*, 46, 521–530.
49. Amati, B., Pick, L., Laroche, T. and Gasser, S.M. (1990) *EMBO J.*, 9, 4007–4016.
50. Sykes, R.C., Lin, D., Hwang, S.J., Framson, P.E. and Chinault, A.C. (1988) *Mol. Gen. Genet.*, 212, 301–309.
51. Käs, E. and Chasin, L.A. (1987) *J. Mol. Biol.*, 198, 677–692.
52. Bouliskas, T., and Kong, C. F. (1993) *J. Cell Biochem.* 53, 1–12.
53. Gasser, S.M., Amati, B.B., Cardenas, M.E. and Hofmann, J.F.-X. (1989) *Int. Rev. Cytol.* 119, 57–96.
54. Hofmann, J.F.-X., Laroche, T., Brand, A.H. and Gasser, S.M (1989) *Cell* 57, 725–737.
55. Amati, B. and Gasser, S.M. (1990) *Mol. Cell. Biol.* 10, 5442–5454.
56. Cockerill, P.N., Yuen, M.H., Garrard, W.T. (1987) *J. Biol. Chem.*, 262, 5394–5397.
57. Phi-Van, L. and Strätling, W.H. (1988) *EMBO J.*, 7, 655–664.
58. Roberge, M. and Gasser, S.M. (1992) *Mol. Microbiol.*, 6, 419–423.
59. Phi-Van, L., Jeanteur, P., Strätling, W.H., Kuchino, Y., Müller, W.E.G. and Paine, P.L. (1990) In *Progress in molecular and subcellular biology*. Springer Verlag, Berlin, Vol. 11, pp. 1–11.
60. Schöffl, F., Schröder, G., Kliem, M. and Rieping, M. (1993) *Transgenic Research* 2, 93–100.
61. Schlake, T. and Bode, J. (1994) *Biochemistry*, in press.
62. Stief, A., Winter, D.M., Straetling, W.H. and Sippel, A.E. (1989) *Nature*, 341, 343–345.
63. Breyné, P., Van Montagu, M., Depicker, A. and Gheysen, G. (1992) *Plant Cell*, 4, 463–471.
64. Allen, G.C., Hall, G.E.Jr., Childs, L.C., Weissinger, A.K., Spiker, S. and Thompson, W.F. (1993) *Plant Cell* 5, 603–613.
65. Mielke, C. (1993) Dissertation, Technische Universität Braunschweig.
66. Zhao, K., Kaes, E., Gonzalez, E. and Laemmli, U.K. (1993) *EMBO J.* 12, 3237–3247.
67. Stockhaus, J., Eckes, P., Blau, A., Schell, J. and Willmitzer, L. (1987) *Nucleic Acids Res.* 15, 3479–3491.