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Nuclear scaffolds and scaffold-attachment regions in higher plants

(chromatin/chromosomes/nuclei/tobacco)

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ABSTRACT **DNA in the nuclei of eukaryotic organisms undergoes a hierarchy of folding to be packaged into interphase and metaphase chromosomes. The first level of packaging is the 11-nm nucleosome fiber, which is further coiled into a 30-nm fiber. Evidence from fungal and animal systems reveals the existence of higher order packaging consisting of loops of the 30-nm fibers attached to a proteinaceous nuclear scaffold by an interaction between the scaffold and specific DNA sequences called scaffold-attachment regions (SARs). Support for the ubiquitous nature of such higher order packaging of DNA is presented here by our work with plants. We have isolated scaffolds from tobacco nuclei using buffers containing lithium diiodosalicylate to remove histones and then using restriction enzymes to remove the DNA not closely associated with the scaffold. We have used Southern hybridization to show that the DNA remaining bound to the scaffolds after nuclease digestion includes SARs flanking three root-specific tobacco genes. This assay for SARs is termed the endogenous assay because it identifies genomic sequences as SARs by their endogenous association with the scaffold. Another assay, the exogenous assay, depends upon the ability of scaffolds to specifically bind exogenously added DNA fragments containing SARs. The tobacco scaffolds specifically bind a well-characterized yeast SAR, and cloned DNA fragments derived from the 3′-flanking regions of the root-specific genes are confirmed to contain SARs by this exogenous assay.**

The structure of the nucleosome core, a complex of eight histones and ≈146 base pairs (bp) of DNA wrapped around the outside, is now fairly well understood (1). Much less well understood is the structure of the 30-nm chromatin fiber and how these fibers are coiled and folded to form interphase and metaphase chromosomes (2). Central to many models of this "higher order" chromatin structure is the concept of domains formed by loops of the 30-nm fibers attached at their bases to a proteinaceous nuclear or chromosome scaffold. Early evidence for such a model came from electron micrographs of histone-depleted chromosomes and nuclei showing loops of DNA spilling out to form a halo (3–7). Mirkovitch *et al.* (8) showed that the loops were not randomly attached to the nuclear scaffold but that specific DNA regions were involved. These regions, which have been called scaffold attachment regions (SARs), have been partially characterized. The binding sites have been mapped to regions generally ranging from 300 to 1000 bp, which are generally A+T-rich.

The domains formed by SAR-bounded loops may have functional, as well as structural, significance. It has long been realized that regions of DNase I-sensitive chromatin, which contain transcriptionally poised genes, are not confined to the genes themselves but rather extend over much larger domains (9–11). These DNase I-sensitive domains have been shown to correspond to SAR-bounded loop domains (12–14). Moreover, inconsistencies in the levels of expression of genes in transgenic animals (ascribed to position effects) have been overcome by using large flanking sequences, which included SARs, in the transforming DNA (15–17). The exact mechanism by which this relief of position effect occurs is unknown, but an attractive hypothesis is that the SARs allow the transforming DNA to form its own chromatin domain. Thus, the transforming DNA would be free of the influences of the chromatin structure of domains into which it has become incorporated.

Even though plant systems are readily amenable to transformation, they too display inconsistencies in transgene expression (18, 19). The use of SARs to overcome these inconsistencies has not been reported in plant systems, but our isolation of a tobacco nuclear scaffold demonstrates the feasibility of such an approach. Our plant scaffold preparations specifically bind a well-characterized yeast SAR and have enabled us to identify endogenous SARs in sequences that flank tobacco genes.

MATERIALS AND METHODS

DNA Constructs. Plasmid GA-1 was constructed by inserting an *Eco*RI fragment containing the yeast autonomously replicating sequence ARS1 and the yeast *N*-(5′-phosphoribosyl)-anthranilate isomerase gene (*TRP1*) from pYRp7 (20) into pJKKmf(−) (21). Plasmid RB7 contains a cDNA for a tobacco protein that is a member of an evolutionarily conserved gene family of membrane channel proteins (22). The cDNA is encoded by the *rb7-5A* gene, which is root specific in its expression (23). Plasmids B7-1 through B7-5 are *Hin*dIII–*Hin*dIII fragments, and pB7-6 is a *Hin*dIII–*Sal* I fragment from the *rb7-5A* gene and flanking regions derived from two overlapping genomic clones, λ5A and λ8D (23), subcloned into pBluescript II SK (Stratagene).

Isolation of Nuclei. Protoplasts (24) prepared from 100 ml of a 4-day-old subculture of tobacco NT1 cells (25) were suspended in 40 ml of nuclear isolation buffer according to Mirkovitch *et al.* (8) with slight modifications [1 M hexylene glycol/1% thiodiglycol/20 mM KCl/20 mM Hepes, pH 7.4/0.5 mM EDTA/0.5% Triton X-100/0.05 mM spermine/0.125 mM spermidine/0.2 mM phenylmethylsulfonyl fluoride/aprotinin at 5 μg/ml (Sigma)/10 μM E-64 (Sigma)] and were disrupted with a Dounce homogenizer. Crude nuclei were collected by centrifugation at 300 × *g* for 10 min, resuspended in 40 ml of nuclear isolation buffer, and layered (in aliquots) on top of equal volumes of 15% Percoll (Pharmacia) in nuclear isolation buffer. These gradients were centrifuged at 600 × *g* for 15 min, and the nuclear pellet was washed three

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Abbreviations: LIS, lithium diiodosalicylate; SAR, scaffold-attachment region; HIB1 and HIB2, halo isolation buffer 1 and 2, respectively.

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times with nuclear isolation buffer without Triton X-100, adjusted to 50% glycerol, and stored at −80°C.

Preparation of Nuclear Halos and Characterization of Proteins. Structures called nuclear halos result when histones are removed from nuclei, and DNA loops, constrained only at their bases by means of attachment to the nuclear scaffold, spill out into the surrounding space (3). To prepare halos, aliquots of nuclei (A_{260} = 50) were stabilized for 10 min at 37°C with 1 mM $CuSO_4$. Twenty-microliter aliquots were extracted with 1 ml of either halo isolation buffer 1 (HIB1) (5 mM Hepes, pH 7.4/2 mM EDTA, pH 7.4/2 mM KCl/0.1% digitonin/0.2 mM phenylmethylsulfonyl fluoride/aprotinin at 5 μg/ml/10 μM E-64) (8) or HIB2 (same as HIB1 but with 100 mM lithium acetate replacing the 2 mM KCl) (26) and with increased concentrations of lithium diiodosalicylate (LIS). Nuclear halos were recovered by centrifugation, washed three times in digestion/binding buffer (20 mM Hepes, pH 7.4/20 mM KCl/70 mM NaCl/10 mM $MgCl_2$/1% thiodiglycol/0.2 mM phenylmethylsulfonyl fluoride/aprotinin at 5 μg/ml/10 μM E-64), and used immediately to prepare nuclear scaffolds or to characterize the associated proteins. Nuclear halo proteins were solubilized in SDS sample buffer, run on 18% SDS/polyacrylamide gels, and stained with Coomassie Blue (27).

Preparation of Nuclear Scaffolds. Nuclear halos equivalent to 2 A_{260} units of nuclei, isolated using HIB2/10 mM LIS, were resuspended in 100 μl of digestion/binding buffer supplemented with 100 units of *Hin*dIII and either *Eco*RI or *Xho* I and incubated at 37°C for 3 hr. The resulting nuclear scaffolds were washed once with digestion/binding buffer and used immediately for the exogenous SAR assay.

Exogenous SAR Assay. Nuclear scaffolds equivalent to 2 A_{260} units of nuclei were incubated for 1 hr at 37°C with 20 ng of end-labeled (Klenow fill-in reaction, ref. 28) DNA fragments and 10 μg of sonicated *Escherichia coli* genomic DNA in a total volume of 100 μl of digestion/binding buffer. The nuclear scaffolds were pelleted at 2000 × *g* for 10 min and washed once with digestion/binding buffer without protease inhibitors. Pellet and supernatant fractions were incubated in 50 μl of lysis buffer (1% SDS/proteinase K at 500 μg/ml/20 mM EDTA, pH 8.0/20 mM Tris·HCl, pH 8.0) for 16 hr at 37°C. Equal counts of end-labeled, input DNA fragments (total), pellet, and supernatant fractions were run on a 1% agarose Tris/acetate/EDTA gel (28). The DNA was fixed by soaking the gel in 1% hexadecyltrimethylammonium bromide (CTAB)/50 mM sodium acetate, pH 5.5, for 1 hr (29). The gel was then dried between paper towels and autoradiographed.

Endogenous SAR Assay. Aliquots of nuclear halos equivalent to 20 A_{260} units of nuclei were incubated in 1 ml of digestion/binding buffer with 1000 units of *Eco*RI or *Hin*dIII for 3 hr at 37°C. Scaffolds with bound DNA fragments were pelleted by centrifugation (2000 × *g* for 10 min) and washed once with digestion/binding buffer. The pellet and supernatant fractions were treated with RNase A at 200 μg/ml followed by proteinase K at 500 μg/ml, and subsequently extracted with phenol/chloroform and precipitated with ethanol. Ten micrograms of each sample and *Eco*RI- or *Hin*dIII-digested total purified tobacco NT1 genomic DNA were run on a 0.7% agarose Tris/acetate/EDTA gel, blotted to Nytran (Schleicher & Schuell), and probed with the appropriate DNA fragment (purified RB7 cDNA insert labeled by the BRL random priming procedure).

RESULTS AND DISCUSSION

The nuclear scaffolds are isolated from tobacco suspension cell nuclei by a modification of the procedures of Amati and Gasser (26) and Mirkovitch *et al.* (8). In this procedure, histones and some nonhistone chromosomal proteins are removed by buffers containing LIS (Fig. 1). As a result, the

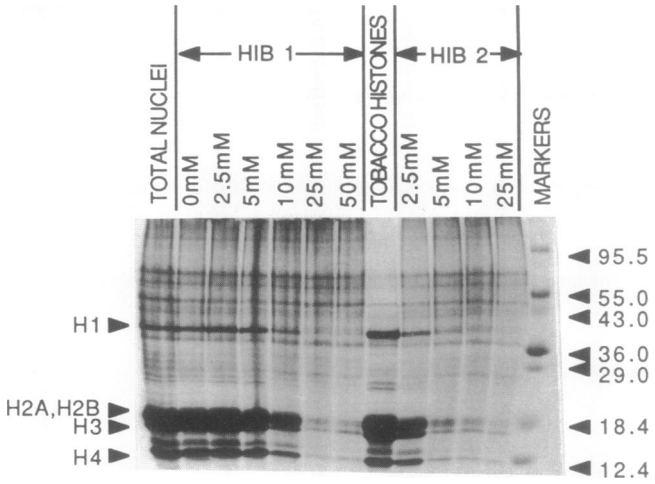

FIG. 1. Histones are removed from nuclei upon treatment with LIS. A Coomassie-stained SDS/polyacrylamide gel of proteins remaining after extracting nuclei with two different halo isolation buffers, HIB1 or HIB2, with increased concentrations of LIS. Other lanes: total nuclei, purified nuclei; tobacco histones, partially purified tobacco histones; and markers, molecular mass markers. Locations of tobacco histones are shown at left, and sizes of markers are indicated at right (kDa). Both HIBs yield similar protein patterns, although a much lower concentration of LIS is required for the depletion of histones for HIB2. Scaffolds isolated with either buffer specifically bind DNA fragments containing SARs (comparative data not shown).

DNA loses structural constraints and forms a diffuse halo structure that can be visualized by staining with a DNA-specific fluorescent stain (data not shown). Similar structures have been observed in fungal and animal systems (7). Fig. 1 illustrates the effect of increased concentrations of LIS in removing histones and several nonhistone chromosomal proteins from tobacco nuclei. At the LIS concentrations used to make nuclear scaffolds (25 mM for HIB1 and 10 mM for HIB2), nearly all the histones are removed, while many other nuclear proteins remain. Although LIS treatment removes most histones, some residual histones remain, even at the highest concentration used. This is a typical result in other scaffold systems (8, 26, 30, 31).

Because no plant SARs were available to test the specific binding of DNA sequences to the tobacco nuclear scaffold, we first tested a well-characterized scaffold binding sequence from yeast. This sequence, the yeast autonomously replicating sequence ARS1, binds scaffolds obtained from yeast and animals (26, 32). The assay, termed the exogenous assay, involves the incubation of the tobacco scaffold with end-labeled restriction fragments of the ARS1 plasmid in the presence of excess nonspecific competitor DNA (sonicated *E. coli* genomic DNA). In this experiment, exogenous SAR sequences compete with the endogenous bound genomic sequences for binding sites on the scaffold (33). Fig. 2 shows the specific binding of the yeast SAR to the tobacco nuclear scaffold. Only the ARS1-containing fragment partitions with the scaffold pellet, indicating that the tobacco scaffold specifically binds SAR-containing fragments. Three other fragments of various sizes (vector, TRP1, and a small fragment derived from the multiple cloning site) do not contain SARs and do not bind to the nuclear scaffold. Scaffolds isolated at LIS concentrations that do not remove histones fail to specifically bind the ARS1 sequence but instead bind all four fragments nonspecifically (data not shown).

Once we had shown that our tobacco nuclear scaffold specifically binds a yeast SAR, we proceeded to identify SARs from the tobacco genome. To do this we employed the endogenous assay in which Southern hybridization is used to

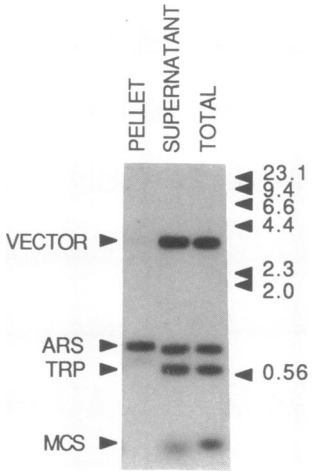

FIG. 2. The yeast ARS1 sequence specifically binds to tobacco scaffolds in the exogenous assay. Plasmid GA-1, carrying an *Eco*RI fragment containing the yeast autonomously replicating sequence ARS1 and the yeast *TRP1* gene, was digested with *Eco*RI and *Hin*dIII, end-labeled, and incubated with tobacco nuclear scaffolds. Lanes: pellet, scaffold-bound fraction; supernatant, unbound fraction; and total, end-labeled *Eco*RI- and *Hin*dIII-digested pGA-1 (input). Arrows at left indicate the following: vector, pJKKmf(−) (3550 bp); ARS1, an 838-bp fragment containing the yeast autonomously replicating sequence; TRP1, a 615-bp fragment containing the yeast *N*-(5′-phosphoribosyl)-anthranilate isomerase gene; and MCS, multiple cloning site from the vector (55 bp). Positions of λ *Hin*dIII markers are shown at right (kb). Only the ARS1 fragment partitions with the scaffold-containing pellet, indicating that this sequence, which acts as a SAR in yeast and animal systems, also acts as a SAR with the tobacco scaffolds.

detect sequences that remain bound to the scaffold after restriction enzyme digestion. Tobacco nuclear scaffolds were prepared by digesting halos with either *Eco*RI or *Hin*dIII followed by centrifugation. Scaffold-associated DNA in the pellet and non-scaffold-associated DNA in the supernatant were isolated and purified. Equal amounts of DNA from these two sources and a control of *Eco*RI- or *Hin*dIII-cut total tobacco genomic DNA were separated on agarose gels and blotted onto Nytran. The blotted DNA samples were probed with a tobacco root-specific cDNA (pRB7) (23), which is encoded by the *rb7-5A* gene. Two other *rb7* genes cross hybridize with the rb7-5A cDNA, yielding weaker signals (Fig. 3*B*, *Eco*RI total lane). Genomic restriction maps of the *rb7-5A* gene and one of the related genes (*rb7-18C*) are shown in Fig. 3*A*. The third *rb7* gene, which has not been cloned, will be referred to as *rb7*-UC for discussion. When halos are digested with *Eco*RI, three bands appear in the pellet lane of the Southern blot (Fig. 3*B*, *Eco*RI lanes). These bands correspond to SAR-containing fragments from all three divergent genes (*rb7-18C*, 10.7 kb; *rb7-5A*, 7.5 kb; *rb7*-UC, 5.5 kb). The bands that hybridize weakly in the *Eco*RI total lane (10.7 kb and 5.5 kb) show strong signals in the *Eco*RI pellet lane. This result indicates that these sequences are highly enriched in the pellet fraction and, thus, contain strong SARs. In contrast, the 7.5-kb fragment from the *rb7-5A* gene partitions between the pellet and supernatant, which may indicate that it contains a weak SAR (see below). *Hin*dIII cuts just outside the coding regions both 5′ and 3′ of all three genes, yielding a similar fragment of 1.8 kb. This *Hin*dIII fragment is present in the supernatant but not in the pellet from the *Hin*dIII digested halos (Fig. 3*B*, *Hin*dIII lanes) and, thus, is not bound to the scaffold. This result is expected because scaffold attachment regions have not been found in coding sequences (7).

To locate the scaffold-binding regions in the flanking sequences, exogenous assays were done using a set of six

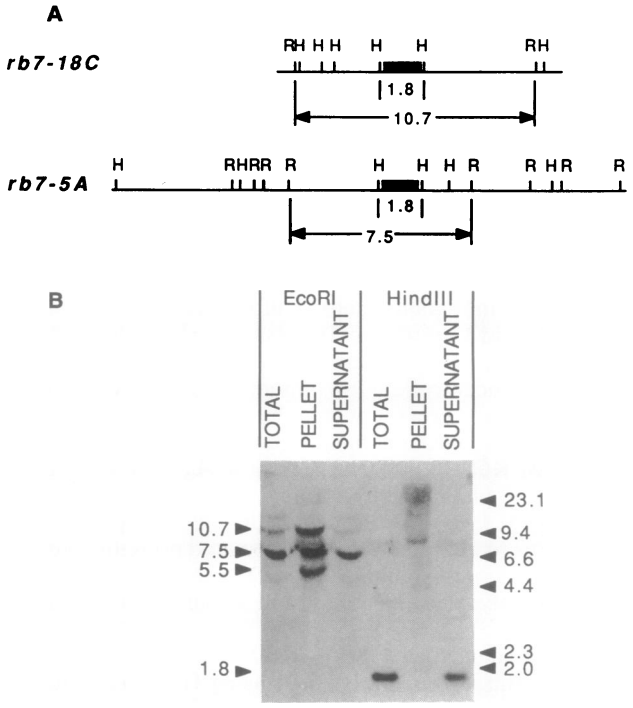

FIG. 3. SAR sequences flank the RB7 tobacco root-specific genes. (*A*) Restriction maps of two tobacco root-specific genes (*rb7-18C* and *rb7-5A*). Location of bands seen on the Southern blot are indicated below the maps (kb). Black box, *RB7* coding region; R, *Eco*RI; H, *Hin*dIII. (*B*) Endogenous assay of SARs that flank the *RB7* genes. Nuclear halos were digested with either *Eco*RI or *Hin*dIII, and the scaffolds were pelleted by centrifugation. DNAs from both pellet and supernatant were purified and subjected to Southern analysis using the RB7 cDNA as a probe. Lanes: total, purified tobacco NT1 genomic DNA digested with either *Eco*RI or *Hin*dIII; pellet, scaffold-bound sequences; and supernatant, released, unbound sequences. Sizes of relevant bands are shown at left of gel (kb). Positions of λ *Hin*dIII markers are indicated at right. In the *Eco*RI lanes, the 10.7-kb fragment from *rb7-18C* and the 5.5-kb fragment from *rb7*-UC partition strongly with the pellet and, thus, contain SARs. The 7.5-kb fragment from *rb7-5A* partitions about equally between pellet and supernatant, a possible characteristic of a weak SAR. In the *Hin*dIII lanes, the 1.8-kb fragment containing the RB7-coding region partitions with the supernatant and, thus, does not contain a SAR.

subclones spanning 23 kb of the *rb7-5A* gene (Fig. 4). These subclones contain *Hin*dIII–*Hin*dIII fragments (pB7-1 through pB7-5) or a *Hin*dIII–*Sal* I fragment (pB7-6) from genomic clones λ5A or λ8D (23) inserted into the multiple cloning site of pBluescript II SK (Stratagene). Plasmids B7-1 through B7-6 were digested with *Hin*dIII, *Xho* I, and additional restriction enzymes as indicated in Fig. 4. The fragments were end-labeled and used in exogenous scaffold binding assays as described in the *Materials and Methods* section. Plasmids B7-1, B7-3, and B7-4 do not contain SAR sequences; all of the fragments partition with the supernatant fraction. Plasmids B7-5 and B7-6 both contain strong SARs; specific restriction fragments partition almost completely into the pellet fraction. The SAR fragment on pB7-5 is completely contained on a 2.6-kb *Sal* I–*Hin*dIII fragment, but when the plasmid is digested with *Eco*RI, a strong-binding 2.7-kb *Eco*RI–*Eco*RI fragment as well as a weak-binding 1-kb *Eco*RI–*Hin*dIII fragment are observed. The binding of the 1-kb fragment is described as weak because the fragment partitions with both supernatant and pellet in the exogenous assay (1-kb fragment in Fig. 4, lanes B7-5, R). A similar situation occurs with pB7-6 where the entire SAR sequence is contained on a 2.7-kb *Eco*RI–*Eco*RI fragment. When this plasmid is digested with *Xba* I, a strongly binding 1.2-kb *Xba*

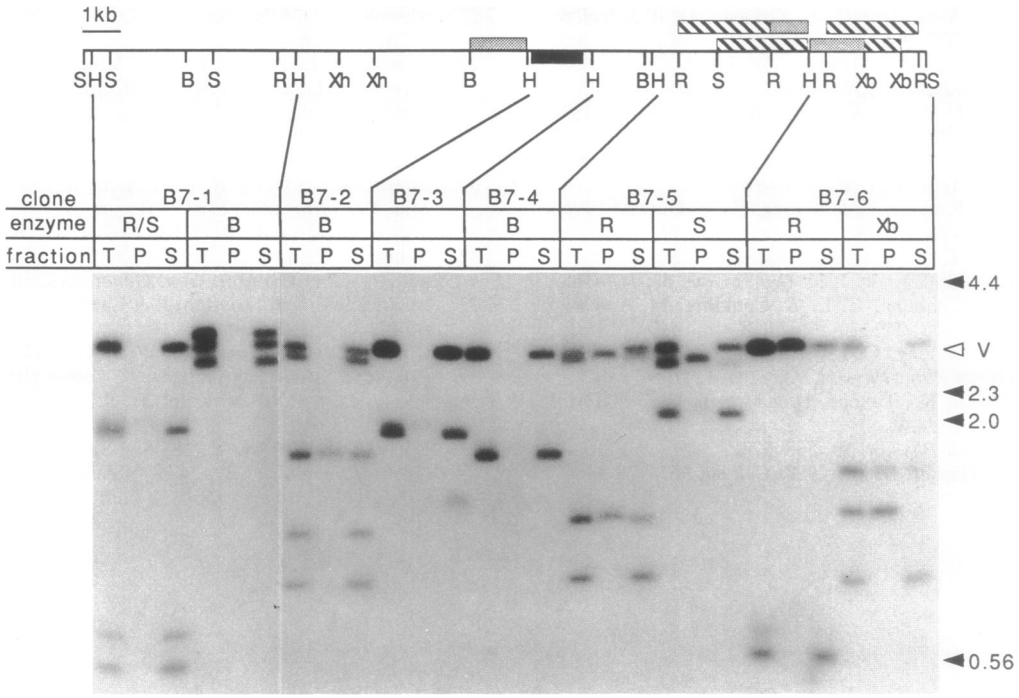

FIG. 4. Mapping of SARs flanking the *rb7-5A* gene with the exogenous assay. A restriction map of the *rb7-5A* gene and location of the subclones used in the assays are shown; only pertinent restriction sites are included. Plasmids B7-1 through B7-6 were digested with *Hin*dIII and *Xho* I, which cleave the insert from the vector. The plasmids were further digested with additional restriction enzymes, as indicated on the line labeled enzyme. The resulting fragments were end-labeled and used in exogenous binding assays, as described. Lanes: T, total end-labeled DNA probe (input); P, pellet fraction (scaffold-bound fragments); and S, supernatant fraction (unbound fragments). Restriction enzymes: B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; S, *Sal* I; Xb, *Xba* I; Xh, *Xho* I. Black box, RB7-coding region; striped boxes, fragments that display strong binding to the nuclear scaffold; stippled boxes, fragments that display weak binding to the nuclear scaffold. Locations and sizes (kb) of λ *Hin*dIII markers (solid arrows) and vector pBluescript II (open arrow) are indicated at right.

I–*Xba* I fragment as well as a weakly binding 1.5-kb *Hin*dIII–*Xba* I fragment are seen. These data suggest that pB7-5 and pB7-6 both contain SAR sequences and that *Eco*RI and *Xba* I, respectively, cut within the SAR to yield partially active fragments. Such an observation is not unexpected. SAR sequences are typically A+T-rich and contain several copies of loosely defined motifs (7). Several lines of evidence indicate that binding does not occur at a particular motif, but rather several copies of these motifs are required for efficient binding (34–37). This result may explain why the *rb7-5A* 3′ SAR(s) can be cut into several fragments resulting in both strongly and weakly binding fragments. Even though the SARs contained on plasmids RB7-5 and RB7-6 are separated by sequences that have no SAR activity, it is difficult to establish whether this region functions as one large SAR or several smaller SARs *in vivo*.

Plasmid B7-2 displays a weak-binding 1.5-kb *Bam*HI–*Hin*dIII fragment that encompasses the *rb7-5A* promoter region. When cut with a variety of restriction enzymes, this region consistently yields weak binding, regardless of the fragment size (data not shown), indicating that the cuts at the *Hin*dIII or *Bam*HI sites have not destroyed a strongly binding SAR. This demonstration of a weak SAR by the exogenous assay is consistent with the observation that in the endogenous assay (Fig. 3) the 7.5-kb band (*rb7-5A* gene) is equally partitioned between pellet and supernatant fractions. The finding that this region binds weakly in both the exogenous and endogenous assays raises the question of how this sequence might be associated with the nuclear scaffold *in vivo*.

In summary, we have prepared plant nuclear scaffolds, which consist of a variety of nonhistone proteins and retain only small amounts of histones. A well-characterized yeast SAR binds specifically to the tobacco nuclear scaffold, and we have identified tobacco SARs flanking three root-specific genes by means of the endogenous assay. We have mapped the SARs in the flanking regions of one of these genes by using an exogenous binding assay. The identification of these sequences will allow further studies of chromatin domains bounded by SARs, and the sequences may be used in plant-transformation experiments to test the ability of plant SARs to form independent chromatin domains and, thus, possibly affect the expression of introduced genes in transgenic plants.

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