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Altered Chromatin Conformation of the Higher Plant Gene Phaseolin†

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ABSTRACT: Micrococcal nuclease, DNase I, and S1 nuclease were used to probe alterations in the chromatin conformation of phaseolin genes which encode the major seed storage proteins of the French bean (*Phaseolus vulgaris* L. cv. Tendergreen). Comparisons were made between cotyledon tissue where the genes are expressed and leaf tissue where the genes are not expressed. The nucleosomal repeat length of leaf chromatin is 191 base pairs (bp). However, in the cotyledon, a tissue which undergoes extensive DNA synthesis leading to endopolyploidy, the repeat length is considerably shorter (177 bp). Phaseolin sequences are organized into the same repeat length as the total chromatin in each respective tissue. Phaseolin genes are preferentially sensitive to DNase I in cotyledons relative to leaves. DNase I hypersensitive sites could not be identified in the flanking DNA of phaseolin genes in chromatin. Although S1 nuclease hypersensitive sites could be identified in DNA sequences flanking the phaseolin genes when analysis was performed on pure, supercoiled DNA, they could not be demonstrated in chromatin.

Abundant evidence derived from animal systems suggests an important role for chromatin structure in the regulation of gene expression [reviewed in Mathis et al. (1980), Elgin (1981), and Weisbrod (1982)]. Transcriptionally active sequences are in an altered conformation which renders them preferentially sensitive to nucleases and particularly to DNase I. Distinct nuclease hypersensitive sites are frequently found in DNA flanking transcriptionally active sequences. High mobility group proteins are preferentially associated with active chromatin and appear in part to be able to confer preferential DNase I sensitivity. In contrast, little is known in higher plants beyond basic levels of chromatin organization [reviewed in Thompson & Murray (1981) and Spiker (1984)]. Only one published study has examined whether transcriptionally active sequences show a modified chromatin structure. In wheat germ, active sequences are preferentially sensitive to DNase I, and proteins apparently analogous to high mobility group proteins are released (Spiker et al., 1983). Few higher plant gene systems have been characterized in sufficient detail to permit more extensive analysis.

We present here the first studies in higher plants on the chromatin conformation of a specific gene. The gene family examined, phaseolin, encodes the major storage protein of the French bean (*Phaseolus vulgaris* L. cv. Tendergreen). Phaseolin expression is under strict developmental control; protein accumulation begins in midmaturation cotyledons and ultimately comprises about 50% of the seed protein (Sun et al., 1978). Phaseolin polypeptides are encoded by a highly conserved multigene family consisting of 7–10 genes, and 7 different genomic clones have been isolated (Hall et al., 1983a; D. R. Talbot et al., unpublished results). Although sexual crosses show phaseolin genes to be tightly linked (Brown et

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0006-2960/84/0423-4225$01.50/0

al., 1981), analysis of a large number of genomic clones indicates a separation of at least 10 kilobase pairs (kbp).[1] The complete nucleotide sequences for one genomic clone (designated 177.4; Slightom et al., 1983) and its corresponding full-length cDNA clone have been determined. The nucleotide sequence of this cDNA clone, as well as five additional full-length cDNA clones, reveals extensive coding sequence homology, but also some slight variation (Hall et al., 1983a). Heteroduplex mapping of two genomic clones (Hall et al., 1983b) suggests that flanking sequence homology does not extend more than about 1000 bp 3′ of the coding sequence. From data herein, we infer that 5′-flanking homology does not extend more than 400–500 bp.

Data presented here show that phaseolin genes are organized in nucleosomes showing the same spacing as total DNA both in cotyledons where they are expressed and in primary leaves where they are not. However, the nucleosome spacing of total chromatin differs in the two tissues. Phaseolin sequences are preferentially sensitive to digestion by DNase I in cotyledon chromatin when compared to leaf chromatin. However, unlike most genes which have been examined, DNase I hypersensitive sites are not detectable in the vicinity of the phaseolin genes in either tissue. Numerous S1 nuclease hypersensitive sites can be identified in DNA flanking phaseolin coding sequences when the DNA is in the form of a supercoiled plasmid. However, we do not detect S1 hypersensitive sites in phaseolin chromatin.

Experimental Procedures

Isolation of Nuclei. Nuclei were isolated by a modification of the techniques described by Spiker et al. (1983) either from primary leaves or from developing cotyledons (8–13-mm stage of development). Tissue was harvested onto ice, and all subsequent operations were performed at 4 °C. The basic isolation buffer containing 10 mM PIPES (pH 7.0), 10 mM NaCl, 5 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride was modified to give the following working buffers: homogenization buffer—1.0 M hexylene glycol, 20% glycerol, and 10 mM $MgCl_2$; Percoll stocks—0.5 M hexylene glycol, 3 mM $MgCl_2$, and the indicated percentages (v/v) of Percoll (Sigma); wash buffer—0.5 M hexylene glycol and 3 mM $MgCl_2$. To aid disruption, leaf tissue was treated with cold diethyl ether for 2 min prior to homogenization (Hamilton et al., 1972) and cotyledonary tissue was minced with razor blades. Leaf tissue was ground in 10 volumes of homogenization buffer by using a Polytron homogenizer (Brinkmann). A mortar and pestle were more effective for disrupting cotyledon tissue. Triton X-100 was added to a final concentration of 1% and the bree filtered sequentially through 200-, 100-μM, and 50-μM nylon screens. Filtrates were underlayered with a 5-mL pad of 90% Percoll. After centrifugation (300*g*, 10 min), nuclei were recovered from the 90% Percoll interface, resuspended in wash buffer, filtered through a 20-μM screen, and layered over a Percoll step gradient (5 mL of 90%, 5 mL of 60%, and 10 mL of 40% Percoll for 20 mL of crude nuclear suspension). After centrifugation (600*g*, 10 min), nuclei were recovered from the 40–60% Percoll interface. Nuclei were washed twice by pelleting (300*g*, 10 min) from wash buffer and suspended in the appropriate digestion buffer (see below).

Final preparations typically consisted of about 80% intact nuclei, 20% free nucleoli, and some cell wall debris. Because no degradation was observed upon sham incubation in the absence of exogenously added nuclease, the nuclear preparations appeared free of endogenous nuclease activities (data not shown). DNA isolated from nuclei was greater than 100 kbp in length.

Nuclease Digestions in Intact Nuclei. DNase I (Boehringer) digestions were performed at 15 °C in 10 mM PIPES (pH 7.0), 10 mM NaCl, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 250 mM sucrose. Micrococcal nuclease (Worthington) digestions were performed at 37 °C in the same buffer supplemented with 1 mM $CaCl_2$. S1 nuclease (Sigma, type III) digestions of nuclei were performed at 37 °C in 30 mM sodium acetate (pH 5.5), 1 mM $ZnCl_2$, 10 mM NaCl, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 250 mM sucrose. S1 nuclease digestion at pH 5.5 rather than at pH 4.5 was used to minimize end nibbling and the preferential digestion of (A + T)-rich regions (Maquat et al., 1981).

Enzyme concentrations were varied, and the time of digestion was held constant at 30 min for all digestions. With DNase I, it was necessary to use a lower incubation temperature and range of enzyme concentrations (0.005–0.64 unit/mL) than are typically used for analogous studies in animal chromatin. Because of expected differences in nuclear ploidy levels, digestions were standardized on the approximate DNA concentration rather than the number of nuclei. Digestions contained 3 A_{260} units/mL of total nucleic acid (corrected for scattering at 320 nm). These values were determined spectrophotometrically from samples after lysis with 1% SDS and 5 mM EDTA and resulted in actual DNA concentrations of about 100 μg/mL. Digestions were terminated by dilution into 20 mM EDTA, 50 μg/mL proteinase K (Boehringer), and 1% sarkosyl. After 1 h at 37 °C, DNA was purified by CsCl–ethidium bromide centrifugation (Murray et al., 1977).

S1 Nuclease Studies on Cloned Sequences. Supercoiled plasmids were isolated according to Birnboim & Doly (1979). Relaxed plasmids were prepared by incubation with 0.5 unit of wheat germ DNA topoisomerase I (Promega-Biotec) per μg of DNA in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, and 50 mM NaCl for 1 h at 37 °C. Samples of supercoiled and relaxed plasmids were incubated (37 °C, 1 h) with S1 nuclease in 20 mM sodium acetate (pH 5.5), 1 mM $ZnCl_2$, and NaCl to provide a final concentration of 50 or 280 mM Na^+. With each cation concentration, S1 nuclease was used, at a ratio of 16 or 1.6 units of S1 nuclease per μg of total DNA (carrier DNA included).

Electrophoresis, Blotting, and Hybridization. DNA samples were separated on 1% agarose gels containing 100 mM sodium acetate (pH 8.0) and 10 mM EDTA for about 16 h at 1 V/cm. The mobilities of fragments resulting from *Hind*III digestion of λ and *Hae*III digestion of *φX174* DNA were used to calculate fragment sizes. DNA was transferred to modified nylon membranes (Genatran, D & L Filter Corp.) after a 7-min treatment in 0.2 N HCl to partially depurinate the DNA by essentially following the technique described by Southern (1975) for transfer to nitrocellulose membranes. Although a variety of hybridization conditions were used, the optimum system was found to be 0.5 M sodium phosphate (pH 7.0), 5× Denhardt's solution (Denhardt, 1966), 0.2% SDS, 10% dextran sulfate, 100 μg/mL calf thymus DNA, 5 mM EDTA, 0.05% low-melting agarose, and (3–5) × 10^5 cpm/mL nick-translated probe prepared from isolated inserts [specific activity

[1] Abbreviations: kbp, kilobase pair(s); bp, base pair(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; T_m, temperature at which half of the nucleotides are base paired.

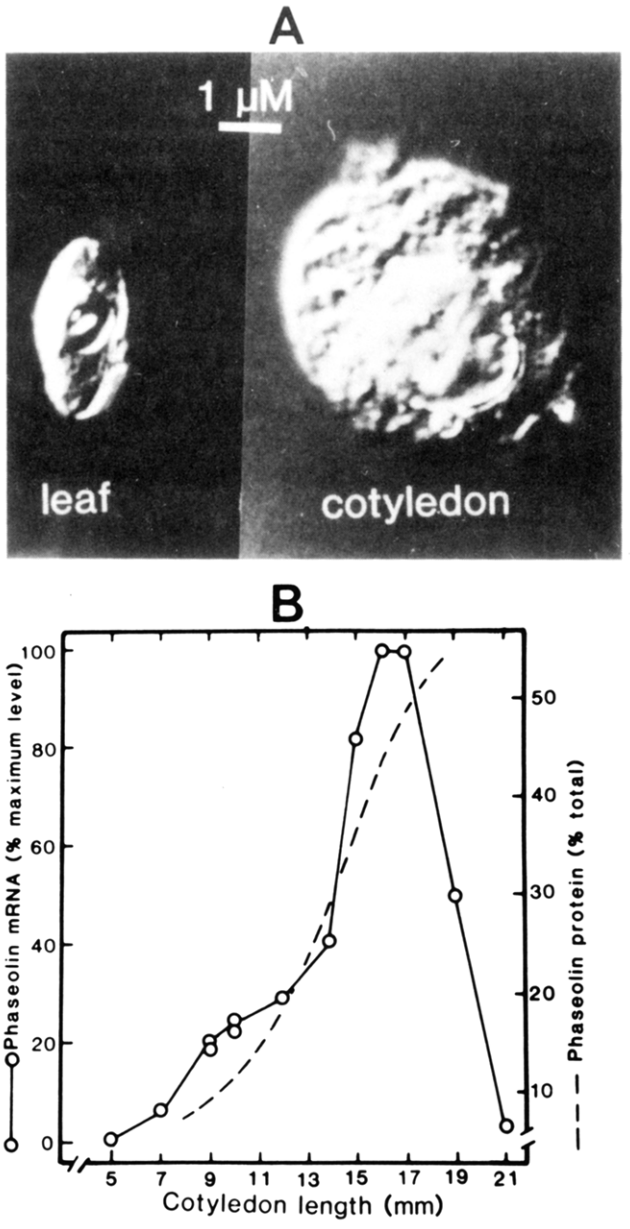

FIGURE 1: (A) Comparison of nuclei from expanding primary leaves and 9-mm-stage developing cotyledons. (B) Onset of phaseolin expression during cotyledon development. The dashed line depicts phaseolin protein accumulation during development as a percent of the total extractable protein [replotted from Sun et al., (1978)]. Steady-state phaseolin mRNA levels are shown by the solid line. Identical quantities of total RNA extracted from cotyledons at the indicated stages were separated on formaldehyde-containing agarose gels, transferred to nitrocellulose (Thomas, 1980), and hybridized with a nick-translated phaseolin cDNA clone probe. After densitometry of nonsaturated autoradiographs, phaseolin mRNA levels were expressed as a fraction of the maximum level of expression. Phaseolin mRNA in 15–17-mm cotyledons constitutes about 40% of the poly(A) RNA (L. M. Hoffman, unpublished results). After this stage, the cotyledons undergo desiccation, resulting in dormancy.

of (0.5–1) × 10^8 cpm/μg]. After 36–48 h at 65 °C, blots were washed extensively at room temperature with 0.12 M sodium phosphate (pH 6.8) and 5 mM sodium pyrophosphate. Blots were washed for 30 min in 0.05 M sodium phosphate at 65 °C (about T_m − 10 °C)before autoradiography for 1–5 days at −70 °C using intensifying screens. Blots were often rehybridized with different probes. To regenerate blots, membranes were tested for 20 min at room temperature in 30 mM NaOH and 5 mM EDTA, rinsed with distilled water, neutralized for 5 min in 50 mM Tris-HCl (pH 7.0), and washed extensively (minimum of three 30-min washes) in 1 mM EDTA (pH 7.0) at 65 °C.

Results

Cotyledon Development and Phaseolin Gene Expression. We compared phaseolin chromatin in expanding primary leaves (where the genes are not expressed) with developing cotyledons (where the genes are expressed). Cotyledon nuclei are generally 2–3 times larger than leaf nuclei and more disperse in size (Figure 1A). This difference is most likely attributable to the fact that cotyledon nuclei are polyploid to varying extents. In legumes, cell division ceases early in cotyledon development, but DNA synthesis continues throughout maturation, resulting in high levels of endopolyploidy. Starch and storage protein syntheses occur primarily after the onset of polyploidy [reviewed in Müntz (1982)]. In French bean, phaseolin mRNA and protein are first detectable at the 7–8-mm stage of development (Figure 1B). Nuclei are very difficult to isolate from 16–17-mm-stage cotyledons wherein phaseolin gene expression is maximal for several reasons: (i) the nuclei are highly polyploid and large and easily destroyed by the forces required to disrupt the tissue; and (ii) the tissue contains large quantities of starch and protein bodies. Consequently, throughout this study, we used 8–13-mm-stage cotyledons which, although not expressing phaseolin genes at maximal levels, can be isolated in sufficient quantity.

Comparisons of Nucleosome Periodicity in Leaf and Cotyledon Nuclei. Samples of nuclei were routinely digested with micrococcal nuclease, and the resulting DNA was analyzed to verify that the gross properties of the chromatin had not been drastically altered during isolation (Figure 2). In the process, it became clear that the average nucleosome repeat length differed in the two tissues. To obtain accurate estimates of the average nuclesome repeat lengths, negatives of the ethidium bromide stained gels were scanned. Fragment lengths calculated from the migration midpoint of each oligomer were divided by the multimer number and averaged. The average unit repeat length based upon this and other gels using two different leaf and three cotyledon nuclei preparations was 191 ± 6 bp (SD) in leaves and 177 ● 7 bp in cotyledons.

The organization of phaseolin genes was compared to that of total chromatin by hybridization with a 3.8 kbp *Bgl*II–*Bam*HI fragment extending from 783 bp upstream of the phaseolin mRNA cap site to 1093 bp downstream of the poly(A) addition site (see Figure 3B, probe C). Densitometric analysis of this blot indicated that the nucleosome repeat length of phaseolin DNA is identical with the total DNA in each tissue. Identical results were obtained when a phaseolin cDNA probe was used to examine the spacing of only the coding sequences (data not shown). Figure 2 also shows that ribosomal DNA reflects the nucleosome organization of the total DNA in each tissue. We note that at this level of analysis, no obvious differences were observed in the rate of micrococcal nuclease digestion of total phaseolin or ribosomal chromatin,[2]

[2] In *Matthiola*, *Brassica*, pea, and wheat, total ribosomal chromatin appears to be slightly less sensitive to nucleases than total chromatin (Leber & Hembleben, 1979; M. G. Murray, unpublished results; W. F. Thompson, personal communication). When the blot used in Figure 3 was hybridized with a ribosomal probe, we observed no detectable loss of ribosomal fragments across the range of DNase digestion examined, suggesting that the bulk of the phaseolin ribosomal genes in bean also is not preferentially sensitive to DNase I. Plant genomes may contain up to 6650 ribosomal genes per haploid genome (Ingle & Sinclair, 1972), and in corn, most ribosomal genes are localized in heterochromatic regions of the nucleolus organizer (Givens & Phillips, 1976). Thus, potential preferential nuclease sensitivity of a small proportion of the transcriptionally active ribosomal genes might be obscured by those that are inactive.

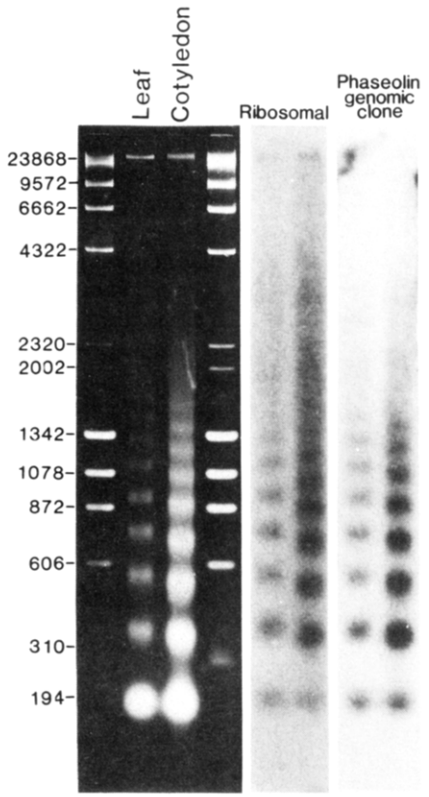

FIGURE 2: Nucleosomal organization of leaf and cotyledon total chromatin, phaseolin genes, and ribosomal genes. Nuclei isolated from primary leaves and 8–11-mm cotyledons were treated with 100 units/mL micrococal nuclease as described under Experimental Procedures, and the resulting DNA was analyzed on 1% agarose gels. After visualization with ethidium bromide, the DNA was transferred and hybridized with a probe corresponding to the entire coding region of one of the phaseolin genes (probe C in Figure 3B). The blot was subsequently regenerated and rehybridized with a pea ribosomal clone probe (Cuellar, 1982). The average nucleosome repeat length is 191 ± 6 bp in leaves and 177 ± 7 bp in cotyledons. ribosomal and phaseolin sequences reflect the total DNA unit repeat present in the tissues from which they were isolated.

either within or between the two tissues.

Preferential DNase I Sensitivity of Phaseolin Genes during Expression. The relative DNase I sensitivity of phaseolin genes was examined by following the degradation of specific restriction fragments using Southern (1975) hybridization. This approach was judged more practical and informative than analysis of phaseolin sequence depletion using reassociation kinetics in solution for several reasons: it was not practical to isolate sufficient quantities of cotyledon nuclei to provide the quantity of DNA needed to drive the reassociation of cloned probes in solution; and because of extensive sequence homology, the DNase sensitivity of individual members of the gene family could not be distinguished in solution.

Samples of leaf or cotyledon nuclei were incubated with varying concentrations of DNase I, after which purified DNAs were restricted with *EcoRI* and *Bam*HI and the fragments separated on neutral agarose gels (Figure 3A). In neither preparation did treatment with up to 0.16 unit/mL DNase I significantly degrade the total DNA restriction profile. The extent of phaseolin sequence degradation was compared by hybridization with a clone spanning the coding sequence (Figure 3C). A simplified diagram of the relevant phaseolin restriction fragments and the probe used is shown in Figure 3B. The multiple fragments observed are attributable to different members of the phaseolin gene family. However, all members contain an *Eco*RI site about 110 bp 3′ from the start of transcription. Fragments labeled on the left of Figure

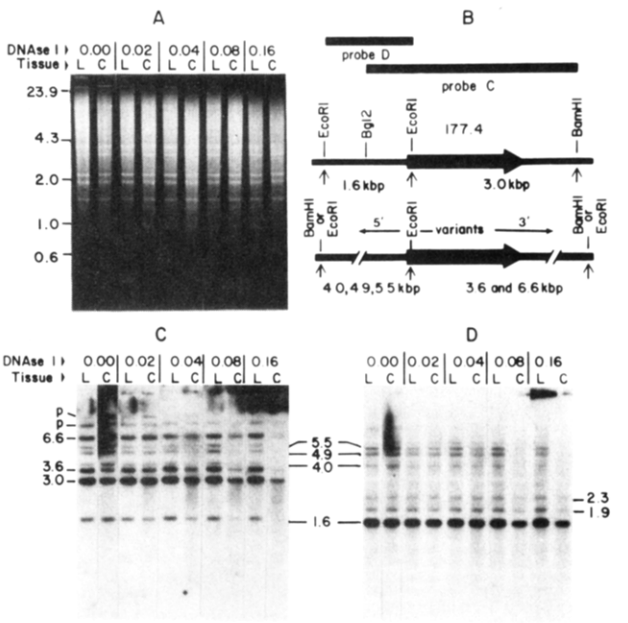

FIGURE 3: Preferential DNase I sensitivity of phaseolin genes in cotyledons compared to that in leaves. (A) Ten micrograms of purified DNA isolated from leaf (L) or 7–11-mm stage cotyledon (C) nuclei that had been incubated with the indicated concentrations of DNase I was digested with *Eco*RI and *Bam*HI and separated on agarose gels. The stained gel shows that the extent of DNase I digestion over the range was similar for each pair and that the overall DNA degradation over the range shown was minimal. (B) A portion of the reference phaseolin genomic clone 177.4 used as a source of probes is shown. Cleavage with *Eco*RI and *Bam*HI divides this gene into a 1.6 kbp fragment containing 5′-flanking sequences and the first 110 bp of the transcript and a 3.0 kbp fragment extending well past the poly(A) addition site. Although the coding regions of all members are very similar, differences in flanking sequences result in additional phaseolin variants 3′ from the *Eco*RI site of 3.6 and 6.6 kbp and variants 5′ from this site of 4.0, 4.9, and 5.5 kbp. The probes used for hybridization are indicated below. (C) Hybridization with a 3.8 kbp *Bgl*II–*Bam*HI fragment (probe C) demonstrates preferential digestion of the bulk of the coding region in cotyledons. No unpredicted small fragments indicative of DNase I hypersensitive sites 3′ to the coding region are evident. Fragments labeled P are attributable to partial digestion. The atypical ratio of fragments in lane 0.00-C is attributable to partial digestion. The fainter bands labeled on the right result from hybridization to the 5′ region of the variants. (D) To search for possible 5′ DNase I hypersensitive sites, the blot was regenerated and hybridized with a probe corresponding to the 5′-flanking region of the gene (probe D). This blot was deliberately overexposed to detect potential cleavage within the 1.6 kbp fragment by DNase I. No distinct fragments smaller than the 1.6 kbp parent fragment suggestive of 5′ hypersensitive sites are evident. Signals on the other variants are too weak to allow definitive conclusions as to hypersensitive sites in other variants. The 2.3 and 1.9 kbp bands are not contiguous with phaseolin coding sequences, and their origins are not understood.

3C are attributable to phaseolin gene variants which have different *Eco*RI or *Bam*HI sites in their downstream-flanking DNA. The 3.0, 3.6, and 6.6 kbp fragments represent 3–4, 1–2, and 3–4 copies per haploid genome, respectively (D. R. Talbot et al., unpublished results). Differences in the relative intensity of hybridization to the various fragments reflect variant gene copy numbers, the extent of homology 3′ from the coding region, and probably also transfer efficiency. Exposure to DNase I concentrations greater than 0.04 unit/mL results in loss of phaseolin sequences in the cotyledon chromatin but has no effect in the leaf chromatin. Identical results were obtained when this blot was rehybridized with a phaseolin cDNA clone (data not shown). Fragments labeled on the right of Figure 3C result from gene variants which have different *Eco*RI or *Bam*HI sites in upstream-flanking DNA. Although the signals are considerably weaker (because a much smaller region is

hybridizing with the probe; see Figure 3B), it is clear that these regions also are preferentially sensitive to DNase I digestion in cotyledon chromatin.

At present, we only have definitive proof that the sequences within a 3.0 kbp *Eco*RI–*Bam*HI fragment encode a phaseolin mRNA. A complete cDNA clone whose sequence corresponds precisely to that of a phaseolin genomic clone carrying this fragment has been isolated (Slightom et al., 1983). Proof that all of the other fragments in Figure 3C represent transcriptionally active phaseolin genes will require matching the sequences of other full-length cDNA clones with the various genomic clones after the latter have been sequenced. At present, we can conclude that all members of the phaseolin gene family are more sensitive to DNase I than is total DNA in the transcriptionally active cotyledon tissue. However, it is premature to attempt quantitative comparisons between the members of the gene family.

DNase I Hypersensitivity. DNase I hypersensitive sites are generally found in the 5′-flanking DNA and often in the 3′-flanking regions of transcriptionally active genes (Elgin, 1981; Weisbrod, 1982). A hypersensitive site located in the 3′-flanking region of the phaseolin gene present on the 3.0 kbp *Eco*RI–*Bam*HI fragment (Figure 3C) should result in the appearance of additional fragments smaller than 3.0 kbp. Although a faint indication of degraded material is evident below the 3.0 kbp fragment, no distinct fragments were observed. We cannot exclude the possibility of 3′ hypersensitive sites flanking the other phaseolin genes because smaller fragments might be obscured by the major parent phaseolin-hybridizing fragments.

To detect DNase I hypersensitive sites in the 5′-flanking regions of phaseolin genes, a probe extending from 110 bp inside the gene to 1471 bp upstream from the cap site was used (Figure 3B, probe D); while this probe would not permit precise mapping of potential hypersensitive sites, it would reveal their presence. Hybridization to the corresponding 1.6 kbp genomic fragment is much stronger than to the fragments containing the upstream-flanking regions of the other phaseolin gene variants because flanking sequence homology does not extend more than 400–500 bp upstream. This conclusion is based on the fact that similar levels of hybridization to all upstream-flanking regions were observed when a probe extending only 422 bp upstream from the transcription start was used (see Figure 5). Even after prolonged autoradiography, we did not detect any subfragments below 1.6 kbp which would indicate hypersensitive sites in any sample. In Figure 3D, parent fragment signals were too weak to definitely exclude the existence of hypersensitive sites in the 5′-flanking DNA of the other phaseolin variants. However, in additional experiments, DNAs were restricted with *Bam*HI and *Bgl*II and probed with either a cDNA clone or the 3.8 kb genomic subclone to ensure uniformly strong hybridization of all variants and to eliminate the possibility that cleavage of a small fragment that may not bind to the membrane would be missed (data not shown). Because these experiments also failed to detect any specific cleavage, we conclude that there is no evidence for DNase I hypersensitive sites in the vicinity of active phaseolin genes.

S1 Nuclease Hypersensitivity. S1 nuclease has also been used as a probe for changes in DNA conformation (Lilley, 1981; Larsen & Weintraub, 1982; Burch & Weintraub, 1983). In active globin chromatin, several S1 hypersensitive sites have been indentified which show reasonable correspondence to the DNase I hypersensitive sites in chromatin and to S1 hypersensitive sites in supercoiled plasmids containing globin sequences (Larsen & Weintraub, 1982).

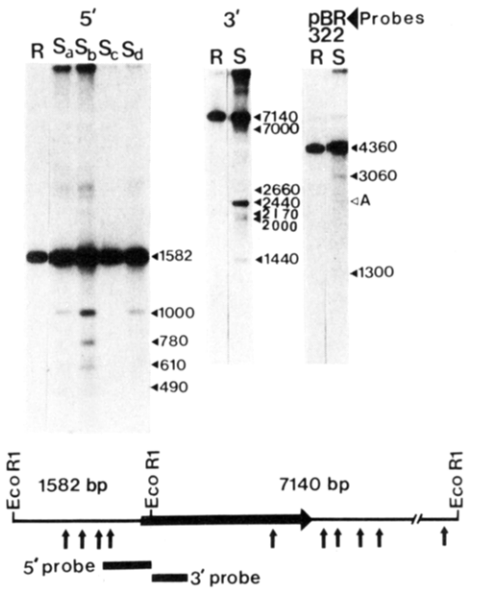

FIGURE 4: S1 hypersensitive sites in supercoiled phaseolin DNA. An 8.8 kbp subclone in pBR322 of the phaseolin genomic clone 177.4 was examined. Samples of either substantially supercoiled (S) or relaxed plasmids (R) were exposed to S1 nuclease prior to restriction with *Eco*RI, blotting, and hybridization with probes which would enable mapping of cleavage sites relative to the *Eco*RI reference site within the gene by using the approach of Wu (1980). The probes used and identified sites are diagrammed below. A pBR322 probe was also used to compare the extent of cleavage of the known S1 hypersensitive sites in the vector to those in the phaseolin sequence. The effect of different S1 digestion conditions is shown only in the supercoiled case by using the indicated 5′ probe to position sites upstream of the coding sequence: (S_a) 1.6 units of S1/μg of DNA, 50 mM Na^+; (S_b) 16 units/μg, 50 mM Na^+; (S_c) 1.6 units/μg, 280 mM Na^+; (S_d) 16 units/μg, 280 mM Na^+. The lower salt ratio and higher S1 nuclease ratio produced the most prominent series of subfragments (480, 610, 780, and 1000 bp) below the parent 1582 bp fragment, and all other lanes on this figure show the result of this treatment. Relative to the start of transcription, they correspond to a weak region at −380 bp and three distinct sites at −500, −670, and −890 bp. In an analogous fashion, five sites could be identified downstream of the reference *Eco*RI site by using the 3′ probe (see text). In this case, the parent fragment is about 7.2 kbp. The pBR322 sequence in this clone is cleaved only weakly, resulting in 3060 and 1300 bp subfragments which would correspond to recognition of the "major" site previously identified in this vector (Lilley, 1981). The fragment identified as A is an artifact attributable to incomplete regeneration of the blot prior to rehybridization with pBR322.

Figure 4 shows an analysis of S1 hypersensitive sites in an 8.8 kbp pBR322 subclone of the phaseolin genomic clone 177.4. Samples of supercoiled and relaxed plasmids were treated with S1 nuclease, followed by restriction with *Eco*RI, electrophoresis, and blotting. S1 hypersensitive sites were mapped relative to the *Eco*RI site within the gene by using the indirect end-labeling approach of Wu (1980). Throughout this work, all samples were examined at two S1 nuclease to DNA ratios and at two salt concentrations. On the basis of previous S1 mapping of phaseolin transcripts (Slightom et al., 1983) and the fact that the sequence contains very (A + T)-rich sequences, the pH 5.5 digestion conditions of Maquat et al. (1981) were used. Maximum cleavage was observed with 16 units/μg of DNA in 50 mM Na^+, and the hierarchy of site recognition was unchanged by the conditions used. No S1 nuclease cleavage was ever observed with relaxed plasmids. The reported sites were observed in three replicate experiments.

Using an *Nco*I–*Eco*RI probe extending from the *Eco*RI reference site within the gene to 422 bp 5′ from the transcription start, we detected S1 cleavage 500, 670, and 890 bp

upstream from the start of transcription. A very weak S1 hypersensitive region occurs 380 bp upstream from the transcription start. To map sites downstream from the gene, the blot was regenerated and hybridized with a probe extending from the reference *Eco*RI site to a *Pst*I site 451 bp in the 3′ direction. S1 hypersensitive sites were detected 120, 290, 560, 780, and 5120 bp downstream from the poly(A) addition rate. An additional site was mapped within the fifth intron of the transcribed region. Finally, to compare the extent of cleavage of the sites in the phaseolin sequence with the known sites in pBR322, the blot was regenerated and hybridized with pBR322. The major S1 hypersensitive site in pBR322 (Lilley, 1981) was detected but much less frequently than the sites in the phaseolin sequence. S1 hypersensitive sites were also mapped in supercoiled plasmids containing three other phaseolin gene variants (data not shown). Although reasonable correspondence exists among all four variants in the position of the site within the coding region (occurring in the fifth intron) and those 3′ from the coding sequences, there is no correspondence in the position of 5′ S1 sensitive sites.

The nucleotide sequence of phaseolin clone 177.4 is known from 1471 bp 5′ of the start of transcription to 1093 bp 3′ of the poly(A) addition site (J. L. Slightom, unpublished results). This region was examined for distinctive sequences which have been reported in other systems to assume an S1 sensitive conformation when supercoiled. We find no correlation between the sites of cleavage and either alternating purine–pyrimidine Z-DNA tracts (Singleton et al., 1982), closely spaced direct repeats which could form "slippage" structures (Mace et al., 1980), or homopurine–homopyrimidine tracts (Schon et al., 1983). However, a reasonable correlation can be drawn between the observed sites and inverted repeats which can form S1-sensitive cruciform structures (Lilley, 1981; Mizuuchi et al., 1982). The sequenced region contains 23 inverted repeats [distributed preferentially in (A + T)-rich regions] according to the following criteria: a minimum of 8 bp and 16 hydrogen bonds in the stem; a maximum of 10 bp in the loop; limited A-C and G-T mispairing (Lilley, 1981). Seven of the eight hypersensitive sites in the sequenced region occur within 20 bp of the center of an inverted repeat. No good correlation could be found with the sensitive region closest to the start of transcription. Because S1 cleavage can occur at the base of the stem as well as within the loop (Goding & Russell, 1983), all but one site fall within the limits of electrophoretic measurements of an inverted repeat, but proof will require precise determination of the site of cleavage. However, should inverted repeats prove to be the basis of cleavage, our observation that the sites within the phaseolin sequence are recognized more frequently than those in the pBR322 vector may reflect the higher A + T composition of the former. Moreover, because only some of the inverted repeats may have formed S1 sensitive structures, an altered conformation in the supercoiled state must also be affected by surrounding sequences.

Before attempting proof of the structural basis of S1 nuclease recognization in supercoiled DNA, it was considered more critical to determine whether S1 sensitive sites occur in active chromatin, as has been observed in several animal systems (Larsen & Weintraub, 1982; Burch & Weintraub, 1983). Leaf or cotyledon nuclei were incubated with S1 nuclease, after which analysis of hypersensitive sites followed the approach used in the plasmid studies. That S1 nuclease was active during these experiments could be shown by digesting mixtures of supercoiled plasmid and nuclei (data not shown). In Figure 5, we observe the four parent fragments resulting from upstream phaseolin gene variants but no subfragments

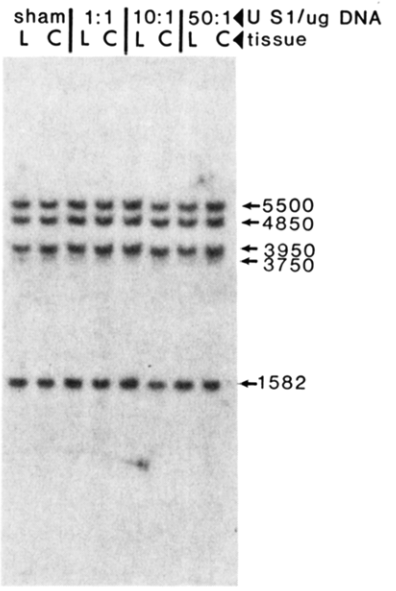

FIGURE 5: Lack of detectable S1 hypersensitivity in phaseolin chromatin. Nuclei isolated from expanding primary leaves of 11–14-mm cotyledons were treated with varying ratios of S1 nuclease. After purification, DNAs were digested with *Eco*RI prior to electrophoresis and blotting. The blot was hybridized with the 5′ probe used to map 5′ hypersensitive sites in supercoiled plasmids (see Figure 4 legend). The labeled fragments reflect phaseolin gene variants which differ in 5′-flanking sequences. In no case were subfragments indicative of S1 hypersensitive sites in chromatin observed.

indicative of S1 cleavage. Variations on this experiment using the digestion conditions of Larsen & Weintraub (1982) and/or higher specific activity probes produced identical results. Thus, although S1 nuclease recognizes specific regions of phaseolin sequences in supercoiled plasmids, it does not do so when these sequences are packaged in either transcriptionally active or inactive chromatin.

Discussion

The phaseolin genes are the first higher plant genes for which alteration in chromatin conformation as detected by preferential DNase I sensitivity can be correlated with the onset of transcription. The DNase I sensitive conformation appears to extend at least from about 1400 bp 5′ to about 1000 bp 3′ from the coding region of phaseolin gene variant 177.4. Because this phaseolin gene used as a source of probes throughout this study is flanked by repetitive sequences outside these boundaries, it was not possible to extend the analysis further. As more information becomes available on the flanking sequences of the other phaseolin genomic clones, it may be possible to extend the analysis of the DNase sensitive region further. In the single previous study of higher plants, transcriptionally active sequences were found to be more sensitive to DNase I digestion than was total DNA (Spiker et al., 1983). However, it was not possible in the latter study to distinguish whether gene-coding sequences in higher plants are always DNase I sensitive or only sensitive when they are active or potentially active. Thus, our demonstration that in plants, as in animals, chromatin structure does change is significant.

Unlike most animal studies, we could find no evidence that the phaseolin genes are flanked by nuclease hypersensitive sites. Although we could identify S1 nuclease hypersensitive sites in the regions flanking four different phaseolin genes when the sequences were analyzed in supercoiled plasmids, we could not identify S1 hypersensitive sites in chromatin. Consequently, at present we cannot speculate that the cleavage sites

identified in plasmids are of any biological significance. Observations such as ours, as well as a recent demonstration that sequences which may form cruciform structures in isolated supercoiled plasmids may not necessarily do so in vivo [e.g., see Sinden et al. (1983)], suggest caution in extending observations made in plasmids or conclusions based on the nucleotide sequence to the level of chromatin conformation and biological function.

Because no comparable information in higher plants is available, it is impossible to assess the extent to which nuclear isolation and digestion conditions may have obscured the presence of real nuclease hypersensitive sites. The buffering conditions used throughout were based on numerous studies in animal sytems wherein nuclease hypersensitivity was evident [e.g., see Stalder et al. (1980)], though the pH during isolation and digestion of nuclei was lower (pH 7.0 instead of 8.0). However, at least with DNase I, we think it unlikely that conditions which permit the detection of general DNase sensitivity would not detect hypersensitivity.

An unexpected and intriguing result of this study was that the nucleosome spacing in cotyledon and leaf chromatin differs. Although differences in nucleosome spacing have been observed in different animal tissues [e.g., see Morris (1976)] as well as during the activation of specific animal genes [e.g., see Smith et al., (1983)], they have not previously been observed in the limited number of plants examined [reviewed in Thompson & Murray (1981) and Spiker (1984)]. For several reasons, it is unlikely that the differences we observe in bean merely reflect differences in the transcriptional activity of the two tissues: expanding primary leaves and developing cotyledons are both transcriptionally very active (De Villiers & Ashton, 1977; Walbot, 1973); the fraction of transcribed DNA in the bean genome is probably quite small and would not make a noticeable contribution of the pattern observed in total DNA [see discussion in Murray et al. (1981)]; and the phaseolin gene specifically does not deviate from the organization of the total DNA when it is in a transcriptionally active state.

We speculate instead that ongoing DNA replication may be involved in the reduction in nucleosome repeat length observed in cotyledon tissue. Transient reductions in nucleosome spacing have been observed in several animal systems during DNA replication [see Lilley & Pardon (1979) and references cited therein], and recently, a short repeat length has been correlated with a rapid increase in DNA synthesis in sea urchin embryos (Chambers et al., 1983). A very significant difference in the two tissues compared here is that, whereas the cotyledon tissue is undergoing extensive DNA endoreduplication, DNA synthesis has ceased in the expanding leaf. Because polyploid nuclei isolated from mature quiescent pea cotyledons that have ceased DNA replication show the same nucleosome repeat as do leaf or embryo nuclei (M. G. Murray, unpublished results), a shortened repeat does not appear to be an inherent property of chromatin in polyploid plant nuclei. We are not aware of any other data on nucleosome spacing during the processes of polyploidization or polyteny in either plants or animals. Detailed analyses of nucleosomal spacing throughout the period of endoreduplication into the period when DNA synthesis ceases are clearly needed. It would also be of interest to examine whether changes in nucleosome repeat length involve different histone variants.

Polyploidy in plant storage tissues is a very common phenomenon [reviewed in Müntz, (1982)]. At present, there has been no suggestion that storage protein genes undergo differential amplification. Because we observe similar levels of phaseolin or ribosomal gene hybridization to equivalent amounts of leaf and cotyledon DNA, we conclude that neither is differentially amplified. Because storage protein synthesis quite frequently follows the onset of polyploidy, one may speculate that alteration of normal chromatin structure resulting from DNA synthesis could in part be involved in the regulation of storage protein gene expression. In such an event, one might expect many details of active storage protein chromatin to differ from most genes that have been examined. Therefore, it will be useful to examine chromatin organization of plant genes which can be induced in one tissue type by treatments such as light or hormones and which might be expected to follow more closely the patterns that have emerged in animal systems. At present, the possible involvement of DNA synthesis in the activation of storage protein genes is not easily approachable in developing seeds. A plant tissue culture system in which storage protein synthesis could be induced and DNA replication modulated would provide a useful model system for detailed examination of this question.

Acknowledgments

We are indebted to J. L. Slightom, M. J. Adang, D. R. Talbot, M. W. Yeazel, R. E. Cuellar, W. F. Thompson, and S. Spiker for providing clones and/or useful discussion during this study. We thank T. C. Hall and C. Sengupta-Gopalan for critical comments on the manuscript.

Registry No. Micrococcal nuclease, 9013-53-0; DNase I, 9003-98-9; S1 nuclease, 37288-25-8.

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γ and γ' Chains of Human Fibrinogen Are Produced by Alternative mRNA Processing†

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ABSTRACT: cDNAs and the genomic DNA coding for the γ and γ' chains of human fibrinogen have been isolated and characterized by sequence analysis. The cDNAs coding for the γ and γ' chains share a common nucleotide sequence coding for the first 407 amino acid residues in each polypeptide chain. The predominant γ chain contains an additional four amino acids on its carboxyl-terminal end (residues 408–411). These four amino acids, together with the 3′ noncoding sequences, are encoded by the tenth exon. Removal of the ninth intervening sequence following the processing and polyadenylation reactions yields a mature mRNA coding for the predominant γ chain. The less prevalent γ' chain contains 20 amino acids at its carboxyl-terminal end (residues 408–417). These 20 amino acids are encoded by the immediate 5′ end of the ninth intervening sequence. This results from an occasional processing and polyadenylation reaction that occurs within the region normally constituting the ninth intervening sequence. Accordingly, the gene for the γ chain of human fibrinogen gives rise to two mRNAs that differ in sequence on their 3′ ends. These mRNAs code for polypeptide chains with different carboxyl-terminal sequences. Both of these polypeptides are incorporated into the fibrinogen molecule present in plasma.

Fibrinogen is a plasma glycoprotein that participates in the final phase of blood coagulation. It is composed of three pairs of nonidentical polypeptide chains, designated α (M_r 66 000), β (M_r 52 000), and γ (M_r 46 500) (McKee et al., 1966), and these chains are held together by interchain and intrachain disulfide bonds (Blomback & Blomback, 1972).

When isolated from plasma, fibrinogen is heterogeneous in charge and size (Mosesson et al., 1972; Henschen & Edman, 1972; Mosher & Blout, 1973). This heterogeneity is due in part to the presence of a minor variant form of the γ chain, called γB (Francis et al., 1980) or γ' (Wolfenstein-Todel & Mosesson, 1980, 1981). The γ' chain has a higher molecular weight than the γ chain, and in human fibrinogen, γ' constitutes about 11% of the total γ-chain population (Mosesson et al., 1972). In rodents, the γ' may be as high as 30% of the total γ-chain population (Legrele et al., 1982). Wolfenstein-Todel & Mosesson (1980) have shown that the differences between the human γ and γ' chains resides in the carboxyl-terminal region of the polypeptide chain. Amino acid sequence analysis has demonstrated that the carboxyl-terminal four amino acid residues of the regular γ chain are substituted by a peptide of 20 amino acids in the γ' chain (Wolfenstein-Todel & Mosesson, 1981). This difference in amino acid sequence accounts for the size and charge heterogeneity of the γ' chain. Accordingly, it was proposed that alternative splicing of the precursor mRNA might be responsible for the generation of the γ' chain (Wolfenstein-Todel & Mosesson, 1981). This proposal was supported by the results of Crabtree & Kant

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received April 25, 1984.* This work was supported in part by Research Grants HL 16919 and HL 28598 from the National Institutes of Health. D.W.C. is an Established Investigator of the American Heart Association.

0006-2960/84/0423-4232$01.50/0