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Elicitor-Inducible and Constitutive in Vivo DNA Footprints Indicate Novel *cis*-Acting Elements in the Promoter of a Parsley Gene Encoding Pathogenesis-Related Protein 1

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The presence of three genes encoding pathogenesis-related protein 1 (PR1) in cultured parsley cells and the activation of all three genes by fungal elicitor are demonstrated. In vivo dimethyl sulfate footprinting was used to identify two putative sites of protein-DNA interaction in the promoter of one PR1 gene, located around positions −240 and −130 relative to the transcription start site. The TATA-distal footprint was elicitor dependent and appeared within 30 minutes of elicitor treatment, concomitant with the onset of PR1 transcription. The second footprint was observed irrespective of whether elicitor was present or absent. The two footprinted regions contain, in opposite orientation, nearly identical 11-base pair motifs that are unrelated to any known *cis*-acting element in elicitor-activated or pathogen-activated plant genes. The results demonstrate the usefulness of in vivo footprinting for the identification of *cis*-acting elements within promoters not accessible to other types of analysis.

INTRODUCTION

Activation of several defense-related genes is involved in the response of plants to pathogen attack (Darvill and Albersheim, 1984; Hahlbrock and Scheel, 1987). Cultured parsley cells treated with a cell wall elicitor derived from the fungus *Phytophthora megasperma* f sp *glycinea* (*Pmg*) have been studied extensively as a model system for the analysis of these reactions (Kombrink et al., 1986). Best characterized are the genes coding for two enzymes of phenylpropanoid metabolism, phenylalanine ammonia-lyase and 4-coumarate:CoA ligase, that are activated by elicitor as well as UV light (Kuhn et al., 1984).

Recently, several solely elicitor-responsive parsley genes have been identified (Somssich et al., 1989). Among them are the genes encoding pathogenesis-related (PR) proteins 1 and 2 (Somssich et al., 1986). These genes are rapidly activated in cultured parsley cells treated with fungal elicitor and in fungus-infected leaves (Somssich et al., 1986, 1988). One PR gene, a member of the small PR1 gene family, has been isolated and characterized (Somssich et al., 1988).

An important step toward an understanding of the molecular mechanisms of gene activation is the identification of *cis*-acting DNA elements that interact with *trans*-acting regulatory proteins. A variety of methods, including nitrocellulose filter binding, gel mobility shift, and DNase I protection, have been used to detect DNA elements that bind nuclear proteins in vitro (Guiliano et al., 1988; Prat et al., 1989). Although these methods are powerful tools for the study of protein-DNA interactions, the results obtained do not always reflect the in vivo situation (Becker et al., 1986). Mutagenesis experiments, on the other hand, have been used successfully to determine sequences functionally important for gene activation in vivo (Stockhaus et al., 1987; Broglie et al., 1989) but fail to prove directly the interaction of these sequences with proteins.

An alternative method, directly investigating protein-DNA interactions that are established during gene activation, was made available by the development of in vivo dimethyl sulfate (DMS) footprinting (Church and Gilbert, 1984; Nick and Gilbert, 1985). It permits the detection of contact sites of proteins with DNA by way of the enhanced/reduced methylation of guanosine residues in vivo (Nick and Gilbert, 1985). Thus, the methylation pattern of a promoter can be followed during activation of the gene in the living cell, and protein-DNA interactions involved in this process can be monitored directly. The application of this method for plant genes was first reported by Ferl and Nick (1986).

Recently, in vivo footprinting was used to investigate *cis*-acting elements involved in elicitor-mediated activation of a parsley PAL promoter. Three footprints were induced by elicitor treatment, two of which were also detected upon irradiation with UV light (Lois et al., 1989). Sequence comparison revealed the presence of similar elements in the promoters of several other elicitor-activated or UV light-

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activated genes from different plant species, including the two parsley 4-coumarate:CoA ligase genes, as well as some unrelated plant genes (Lois et al., 1989). In contrast, the parsley PR1 promoter contains no similarities to these elements despite the similar expression pattern of the PR1 gene (Somssich et al., 1986).

Here we report the results of in vivo footprinting analyses of the PR1-1 promoter, which define sequences different from the previously reported elements within the promoters of other elicitor-responsive genes.

RESULTS

Isolation of the PR1-2 and PR1-3 Genes

Three types of PR1 cDNA, differing slightly in nucleotide sequence, have previously been isolated, and a gene encoding one of them (PR1-1) has been characterized (Somssich et al., 1988). To gain further information on the whole PR1 gene family, we isolated genomic clones corresponding to the PR1-2 and PR1-3 cDNAs. Figure 1A shows rough restriction maps of 6.5 kb surrounding the coding regions of all three PR1 genes. We used three restriction fragments upstream from the respective coding regions (Figure 1A) as gene-specific probes in genomic DNA blots. Figure 1B shows that each probe detected one genomic EcoRV fragment that can be accounted for by a corresponding fragment on the isolated genomic clones. Hybridization with the PR1-1 cDNA (cPR1), which recognizes the entire family, detected only these fragments. We conclude that no additional PR1 genes are present in the parsley genome. Copy number reconstructions indicate that each of the three genes occurs in one copy per haploid genome (data not shown).

Elicitor-Mediated Activation of the PR1 Genes

To analyze the expression of each individual gene in response to elicitor treatment, we synthesized three gene-specific 20-bp oligonucleotides corresponding to 3′-untranslated regions of these cDNAs. Each of them contained at least three mismatches with respect to the other two. These oligonucleotides were used to probe three identical blots of RNA from untreated and 3-hr elicitor-treated parsley cells (Figure 2A). The specificity of the probes for each of the three mRNAs was verified by their ability to differentiate strongly between the corresponding PR1 cDNAs (Figure 2B) under identical hybridization conditions (Wood et al., 1985). As shown in Figure 2A, the expression of all three PR1 genes is activated by elicitor treatment.

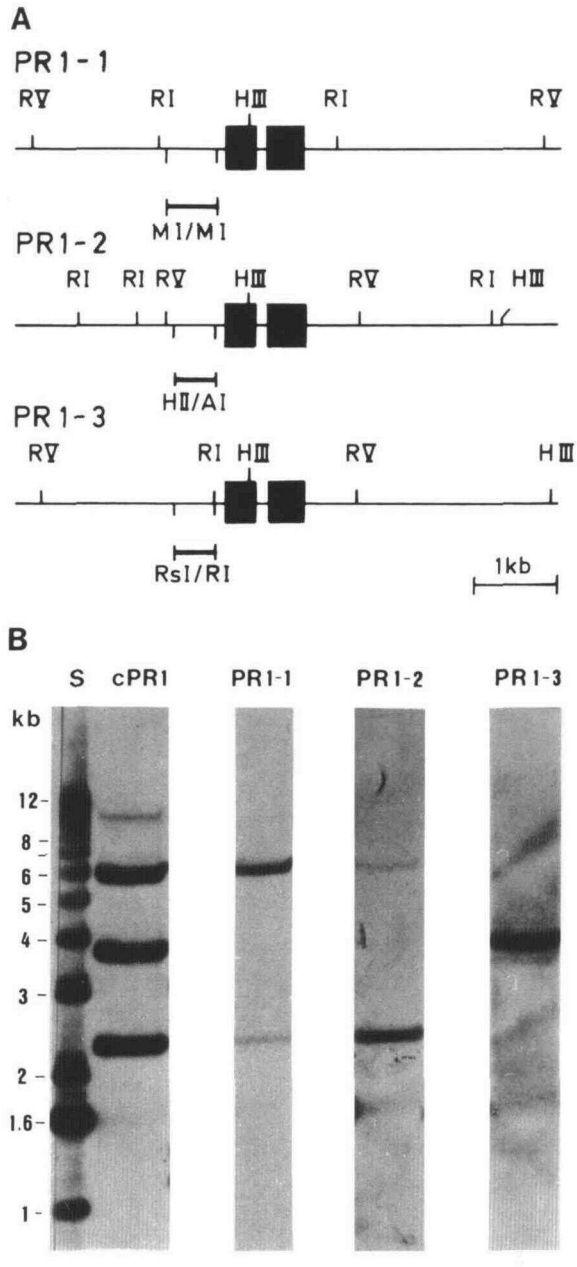

Figure 1. Analysis of the PR1 Gene Family.

(A) Structural organization of the three cloned PR1 genes. Black boxes represent exons; the bars below the lines show the positions and lengths of probes used in **(B)**. Abbreviations of restriction enzymes are: AI, AccI; HII, HindII; HIII, HindIII; MI, MnlI; RI, EcoRI; RV, EcoRV; RsI, RsaI.

(B) Blot hybridization of genomic parsley DNA, restricted with EcoRV. Identical filters were hybridized with the following probes: cPR1-1 (S cPR1), the entire PR1-1 cDNA; PR1-1, 0.8-kb MnlI fragment; PR1-2, 0.53-kb HindII/AccI fragment; PR1-3, 0.46-kb RsaI/EcoRI fragment.

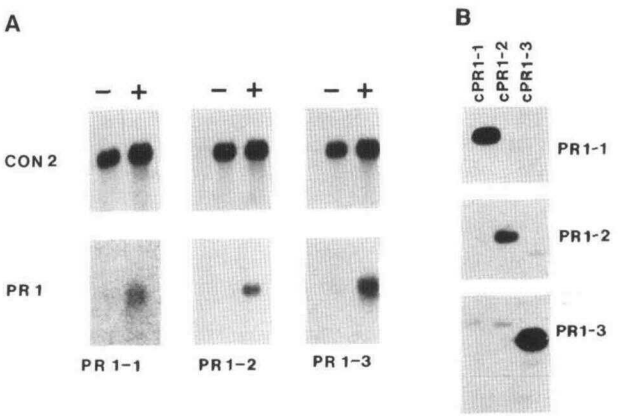

Figure 2. Induction of PR1-1, PR1-2, and PR1-3 mRNA by Elicitor Treatment.

(A) RNA gel blot analysis of PR1 gene expression. RNA blots of 5 μg of poly(A)$^+$ RNA from untreated (–) and 3-hr elicitor-treated (+) cells were hybridized either with a probe representing a constitutively expressed gene (CON 2) to normalize the quantity of mRNA or with gene-specific oligonucleotides (PR1) as indicated below the blot. Note that the blot probed with PR1-1 was exposed twice as long as the others.

(B) Specificity of oligonucleotides. Identical amounts of the three different PR1 cDNAs were excised from plasmids with EcoRI, separated from vector DNA on a 1% agarose gel, and transferred to GeneScreen membrane. Filters were hybridized with gene-specific oligonucleotides as indicated on the right.

In Vivo Footprinting of the PR1-1 Promoter

As a first step toward elucidating the molecular mechanisms involved in the observed gene activation, we searched the promoter of the PR1-1 gene for possible sites of protein-DNA interaction. By in vivo footprinting, we analyzed 500 bp upstream of the transcriptional start site for changes in the accessibility of G residues to methylation and compared the methylation pattern of DNA from elicitor-treated cells with that from control cells, as well as with that obtained with cloned, in vitro methylated DNA. The results are shown in Figure 3A. Around position –240, 8 G residues on both DNA strands showed altered reactivities in the active promoter when compared with the promoter in control cells. Five protected G residues were flanked by 3 residues with enhanced reactivities at the borders of the footprint.

In addition to this inducible footprint, we also detected a constitutive footprint. Around position –130, 3 G residues displayed altered reactivity in vivo relative to cloned, in vitro methylated DNA, regardless of whether or not the cells had been treated with elicitor. The most prominent change was the hyperreactivity of 1 G residue on the coding strand (Figure 3A, far right). To verify that this hyperreactivity was not due to structural differences between genomic and cloned DNA, the cleavage pattern of genomic, in vitro methylated DNA was compared with that of a dilution series of identically treated cloned DNA, as shown in Figure 4. No obvious differences in the in vitro methylation patterns of G residues were detected between genomic and cloned DNA, scanning the coding strand from positions –100 to –200. The hyperreactivity of the G residue at position –128, as observed in vivo, was not seen with in vitro methylated, naked genomic DNA (compare Figure 3A with Figure 4, arrow).

No further significant methylation differences were detected within the 500 bp of the promoter sequence analyzed (data not shown). Visualization of the two footprints was fully reproducible in three independent experiments.

The nucleotide sequences of the footprint-containing regions are shown in Figure 3B. An imperfect inverted repeat was found within the two footprints (long horizontal arrow). Allowing two mismatches, this sequence was not found elsewhere in the PR1-1 gene.

A time-course experiment was performed to follow the appearance of the inducible footprint after addition of elicitor to the cultured cells, as shown in Figure 5. Although no detectable change in the methylation pattern was visible 5 min after onset of the elicitor treatment, the footprint was clearly seen after 30 min and remained visible for at least 5 hr after elicitor application.

DISCUSSION

All available data indicate the existence of three PR1 genes in parsley, all of which are strongly activated by treatment of cultured parsley cells with fungal elicitor. Particularly high induced mRNA levels were observed for PR1-2 and PR1-3. However, whether differences in the individual mRNA amounts are due to distinct rates of transcription or to mRNA stability differences is presently unknown.

Two in vivo footprints were detected in the PR1-1 promoter, each consisting of a confined region containing several G residues with altered sensitivity to methylation. The appearance of the inducible footprint roughly coincides with the elicitor-dependent activation of the PR1 genes. However, although nuclear run-on transcription previously indicated a very rapid increase in the rate of PR1 transcription within 5 min after elicitor application (Somssich et al., 1986), our present data do not show appreciable changes in the methylation pattern at this time point. This apparent discrepancy is probably due to the different methods used. In the nuclear run-on experiments, the parsley cells were not killed immediately after the short treatment with elicitor but were washed and incubated for a further 20 min with a cell wall-degrading enzyme before their disruption. Therefore, the cells were exposed to the elicitor for about 25 min before they were broken open and the nuclei

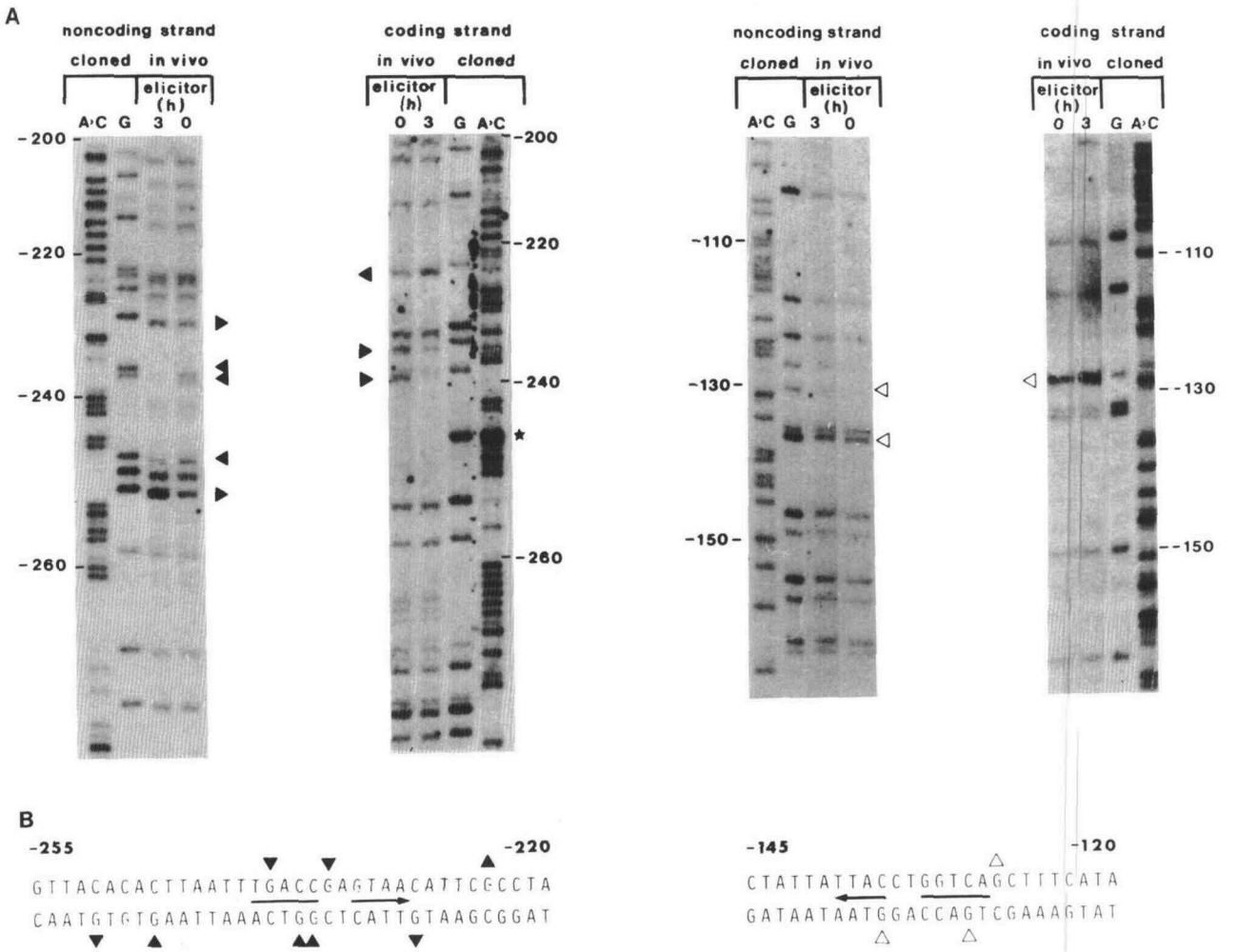

Figure 3. In Vivo Footprints within the PR1-1 Promoter.

(A) In vivo DMS protection. Untreated and 3-hr elicitor-treated parsley cells were exposed to DMS. Genomic DNA sequence G ladders are shown next to sequence ladders for the G and A + C reactions of cloned, in vitro treated DNA. Numbers indicate positions with respect to the transcription start site. Arrowheads pointing toward G residues indicate reduced reactivities, those pointing away from G residues indicate enhanced reactivity. Filled arrowheads represent induced differences, open arrowheads represent constitutive differences. The asterisk indicates an artifactual band within the lanes of the cloned DNA.

(B) Sequence comparison of the footprinted regions. The sequences of the regions analyzed in **(A)** are shown, using the same symbols. The long horizontal arrows indicate an inverted repeat.

isolated. In contrast, addition of DMS in the footprinting experiments immediately kills the cells. Thus, the 30-min time point in this study is most likely to be comparable with the 5-min time point in the previous experiments. Furthermore, we were previously unable to distinguish the three types of PR1 mRNA and cannot exclude the possibility that the precise timing of activation differs for the three PR1 genes.

The nucleotide sequences within the constitutive and inducible footprints showed remarkable similarity, although the methylation enhancement/protection pattern was not identical. Because the two footprints were the only differences found between the in vivo and in vitro methylation patterns within the 500 bp analyzed, their occurrence is unlikely to be due simply to conformational differences between chromosomal and naked DNA. Rather, we as-

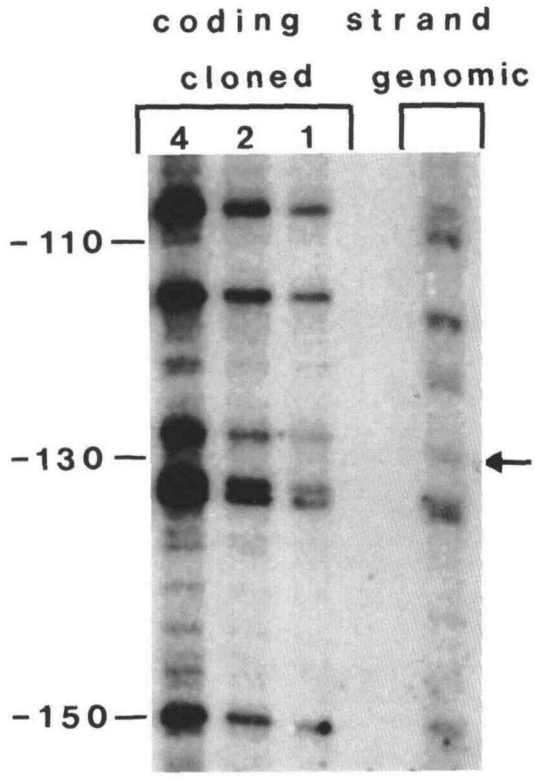

Figure 4. Comparison of in Vitro DMS-Treated, Cloned DNA and Naked Genomic DNA.

The G ladder sequences of the coding strand region from −110 to −150, relative to the transcription start site, are shown. Arrow points to the G residue at position −128 in the genomic DNA lane that shows strong hyperreactivity in vivo (compare with Figure 3A, far right). Onefold, twofold, and fourfold relative concentrations of the cloned DNA were loaded per lane.

sume that the constitutive footprint indicates the binding of protein in both the presence and absence of elicitor, whereas the inducible footprint is probably due to more direct, elicitor-dependent changes in DNA-protein interaction.

In most of the reports to date on in vivo footprinting, the technique has been limited to the comparison of methylation patterns of specific genes in tissues in which they are actively transcribed with those obtained in tissues in which they are transcriptionally inactive (Ephrussi et al., 1985; Becker et al., 1987; Saluz et al., 1988). To our knowledge, this method has been employed in only one case in animals (Becker et al., 1986) and in two cases in plants (Lois et al., 1989; Schulze-Lefert et al., 1989) to follow protein binding during transcriptional activation in vivo. Because the binding we report here was detected within 30 min after elicitor treatment, it occurs more rapidly than previ-

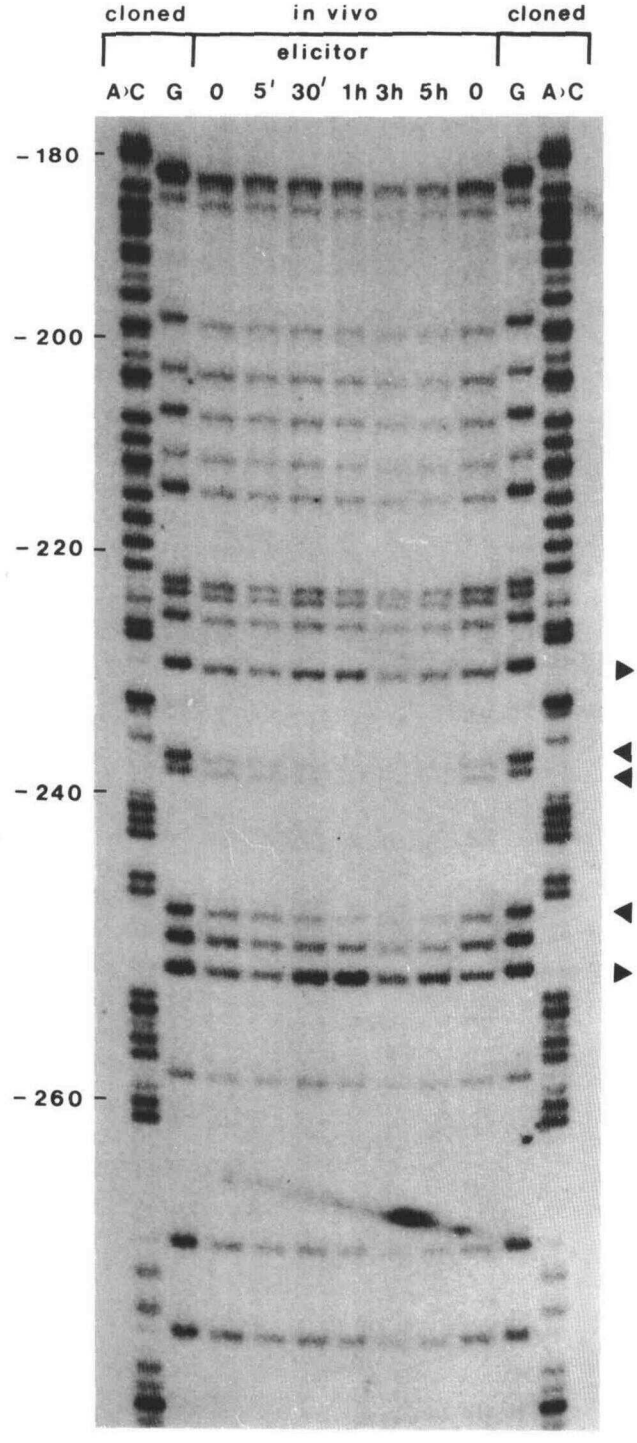

Figure 5. Timing of Elicitor-Induced Changes in the Reactivity of the PR1-1 Promoter to DMS in Vivo.

Parsley cells were treated for various times with elicitor and subsequently exposed to DMS. The noncoding strand between positions −180 and −270 is shown. Symbols are as described in Figure 3.

ously reported for the parsley chalcone synthase and PAL promoters (Lois et al., 1989; Schulze-Lefert et al., 1989) and is comparable with the putative factor binding to the glucocorticoid-responsive element of the tyrosine aminotransferase gene in the hepatoma cell line FTO-2B, reported to appear within 20 min of hormone treatment (Becker et al., 1986). It is open at this stage whether, in further analogy to the activation mechanism of the glucocorticoid-responsive genes, protein binding to the PR1 promoter requires no prior gene activation steps but rather modifications of a preexisting factor.

Earlier results have demonstrated that parsley protoplasts retain their UV light and elicitor responsiveness in several respects (Dangl et al., 1987) and can be used for functional analysis of *cis*-acting elements by transient transformations with reporter gene constructs (Schulze-Lefert et al., 1989; van de Löcht et al., 1990). However, unlike many other elicitor-responsive genes, protoplasting deregulates PR1 gene expression, thus not allowing the functional testing of the footprinted elements of the PR1-1 promoter. This deregulation holds true for all three endogenous PR1 genes as well as for various constructs of the three PR1 promoters in conjunction with the glucuronidase reporter gene, as shown by run-on transcription and transient transformation assays, respectively (data not shown). Therefore, alternative strategies will have to be applied in future studies, including extensive sequence comparison with promoters from other elicitor-responsive genes (Somssich et al., 1989) as well as attempts to identify proteins that interact with putative *cis*-acting elements. Our present results should provide the basis for such approaches.

METHODS

Cell Cultures and Elicitor Treatment

Cell suspension cultures were propagated and treated with elicitor as described (Ragg et al., 1981). *Pmg* elicitor was prepared by the method of Ayers et al. (1976).

Isolation of PR1-2 and PR1-3 Genes

A genomic library, described by Somssich et al. (1988), was used to screen approximately 1.6×10^6 recombinant plaques for hybridization with a PR1-3 cDNA probe (Somssich et al., 1988). Plaques giving a positive signal were purified. The coding regions of three clones containing the PR1 genes in their entirety were sequenced, showing that two were identical to the PR1-2 and one to the PR1-3 cDNAs.

Genomic DNA Blots

DNA isolation, gel electrophoresis, transfer, and hybridization were carried out essentially as described by Douglas et al. (1987).

RNA Isolation and Blotting

Total RNA was prepared according to Lois et al. (1989). Poly(A)$^+$ RNA was isolated using the Pharmacia mRNA purification kit, separated on a 1.2% formaldehyde-agarose gel, transferred to GeneScreen membrane (Du Pont-New England Nuclear) (Kuhn et al., 1984), and fixed by UV cross-linking (Schulze-Lefert et al., 1989).

Gene-Specific Hybridization

The three 20-mer oligonucleotides, 5′-CATAGAGTGCAAAATAATAA-3′, 5′-AAGCCTCAGGCATCTTTGCC-3′, and 5′-AACAGCCTTGAAAATCATAA3′, complementary to sequences within the 3′-untranslated regions of the PR1-1, PR1-2, and PR1-3 cDNAs, respectively (Somssich et al., 1988), were synthesized, end labeled with T4 polynucleotide kinase, and used to probe RNA or DNA blots. Hybridization and washing conditions were as described by Wood et al. (1985).

Cloning

Standard cloning procedures were applied (Maniatis et al., 1982).

In Vivo Footprinting

All steps were conducted essentially as described by Schulze-Lefert et al. (1989). A sucrose-gradient centrifugation step was used to enrich the 1.1-kb EcoRI/HindIII PR1-1 promoter fragment and separate it from the fragments containing the other two PR1 promoters (see Figure 1B). Reference cuts were DdeI (position −415) for the upstream sequence and HindII (position −303) for the downstream sequence. The probes were synthesized as described (Schulze-Lefert et al., 1989), using 18-bp oligonucleotides starting at positions −415 and −303 for the noncoding strand and at −303 and −181 for the coding strand. The synthesis products were restricted to obtain 100-bp to 120-bp probes. Autoradiograms were exposed for 1 week to 2 weeks with intensifier screens at −80°C.

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