The Plant Cell, Vol. 5, 485–496, April 1993 © 1993 American Society of Plant Physiologists

Dissection of the Functional Architecture of a Plant Defense Gene Promoter Using a Homologous in Vitro Transcription Initiation System

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CHS15 is one of a family of bean genes encoding chalcone synthase, which catalyzes the first reaction in a branch pathway of phenylpropanoid biosynthesis for the production of flavonoid pigments and UV protectants and isoflavonoid-derived phytoalexins. The functional architecture of the CHS15 promoter was dissected by a novel homologous plant in vitro transcription initiation system in which whole-cell and nuclear extracts from suspension-cultured soybean cells direct accurate and efficient RNA polymerase II–mediated transcription from an immobilized promoter template. Authentic transcription from the CHS15 promoter template was also observed with whole-cell extracts from suspension-cultured cells of bean, tobacco, and the monocot rice, and the soybean whole-cell extract transcribed several other immobilized promoter templates. Hence, this procedure may be of general use in the study of plant gene regulation mechanisms in vitro. Assay of the effects of depletion of the soybean whole-cell extract by preincubation with small regions of the CHS15 promoter or defined *cis* elements showed that *trans* factors that bind to G-box (CACGTG, −74 to −69) and H-box (CCTACC, −61 to −56 and −121 to −126) *cis* elements, respectively, make major contributions to the transcription of the CHS15 promoter in vitro. Both *cis* element/*trans* factor interactions in combination are required for maximal activity. Delineation of these functional *cis* element/*trans* factor interactions in vitro provides the basis for study of the mechanisms underlying developmental expression of CHS15 in pigmented petal cells established by G-box and H-box combinatorial interactions, and for characterization of the terminal steps of the signal pathway for stress induction of the phytoalexin defense response.

INTRODUCTION

Many plant responses to environmental stimuli or developmental cues involve the selective expression of batteries of functionally related genes (Goldberg et al., 1989; Dixon and Lamb, 1990; Chory, 1991; Katagiri and Chua, 1991). We are studying the transcription of genes encoding flavonoid biosynthetic enzymes as a model system for elucidating the mechanisms underlying the regulation of nuclear gene expression by environmental stimuli and the integration of these responses within developmental programs (Lamb et al., 1989). Flavonoids serve as UV protectants, pigments, and signal molecules, and transcription of flavonoid biosynthetic genes is regulated by light and developmental cues (Hahlbrock and Scheel, 1989). In addition, in bean and other legumes, isoflavonoid-derived pterocarpans function as low molecular weight antimicrobial compounds (Dixon et al., 1983), and the transcription of pterocarpan phytoalexin biosynthetic genes is triggered by wounding, infection, and exposure of cells to biotic or abiotic elicitors (Lamb et al., 1989; Dixon and Harrison, 1990).

The first committed step of flavonoid biosynthesis involves the stepwise condensation of three acetyl units from malonyl-coenzyme A (CoA) with 4-hydroxycinnamoyl-CoA to give naringenin chalcone, which is catalyzed by chalcone synthase (CHS, EC 2.3.1.74) (Hahlbrock and Scheel, 1989). Increased levels of CHS mRNA precede the accumulation of isoflavonoid-derived phytoalexins in elicitor-treated bean and soybean cells and infected plants (Ryder et al., 1984; Bell et al., 1986; Ebel, 1986). Induction of CHS transcription in response to fungal elicitor is observed within 5 to 10 min (Lawton and Lamb, 1987), suggesting the operation of an efficient signal pathway following elicitor perception by putative receptors at the plasma membrane (Cheong and Hahn, 1991; Cosio et al., 1992). Functional analysis of the promoter of CHS15, one of seven bean CHS genes (Ryder et al., 1987), in soybean protoplasts and transgenic tobacco plants has demonstrated that sequences immediately upstream of the coding region, from nucleotide position −326 to +105 relative to the site of transcription initiation, are sufficient for stress induction by elicitors or wounding and developmental expression in pigmented petal cells (Dron et al., 1988; Stermer et al., 1990; O. Faktor, J. Kooter, R. A. Dixon, and C. J. Lamb, unpublished data).

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Characterization of the *cis* elements and *trans* factors involved in CHS15 expression will provide the basis for delineation of the terminal stages of the signal pathways underlying induction by stress stimuli and developmental cues. In similar studies on gene expression in animals and yeasts, the availability of in vitro transcription systems has greatly facilitated the detailed biochemical analysis of gene regulation mechanisms. In this paper, we describe a plant in vitro transcription initiation system that provides efficient, accurate transcription from immobilized DNA templates by soybean nuclear and whole-cell extracts, and we use this homologous system to examine the functional architecture of the CHS15 promoter in vitro.

RESULTS

Development and Characterization of a Plant in Vitro Transcription System

The DNA template used in these studies was a 600-bp fragment excised from the pUC19 plasmid pCHC1-CAT (Dron et al., 1988). This template contains CHS15 sequences from nucleotide position −326 to +105 fused to the 5′ coding sequences of the chloramphenicol acetyltransferase (CAT) reporter gene, as shown in Figure 1. In our initial studies on the development of a plant cell-free transcription initiation system, we observed that a mixture of soybean and HeLa cell extracts accurately and efficiently transcribed the CHS15 template, although neither the plant nor animal cell extract alone was competent with this template (J. A. Arias and C. J. Lamb, unpublished data). By systematically modifying procedures used to prepare active whole-cell extracts from HeLa and yeast cells (Manley et al., 1980; Lue and Kornberg, 1987; Woontner and Jaehning, 1990), we were then able to prepare soybean extracts that efficiently transcribed the CHS15 promoter template, as shown in Figure 2. However, standard transcription reactions with these plant extracts generated a large number of radiolabeled products that hindered the identification and quantification of CHS15 template-specific transcripts (Figure 2, lanes 1 and 3). This background radiolabeling was not dependent on exogenous DNA template, and we reasoned that it might be separated from the products of template-dependent transcriptional activity by the use of an immobilized template system (Arias and Dynan, 1989). In this approach, transcription complexes were assembled on a CHS15 promoter template fragment coupled to agarose beads, washed to remove unbound materials that contribute to background radiolabeling reactions, and incubated with labeled and unlabeled ribonucleoside triphosphates to generate CHS15 transcripts. Washing such immobilized transcription complexes resulted in the almost quantitative recovery of template-dependent transcriptional activity in the near absence of background radiolabeled products (Figure 2, lanes 2 and 4 compared with equivalent unwashed samples, lanes 1 and 3).

In reactions with immobilized CHS15 promoter as the template, the soybean whole-cell extract generated a single major product of 260 nucleotides. This is the expected size for a transcript generated by accurate initiation from the CHS15 promoter and subsequent transcription through to the 3′ terminus of the template. In some reactions, low levels of shorter products appeared as a weak tail originating from the 260-nucleotide band (Figure 2, lanes 4 and 6). Transcription by the soybean whole-cell extract was template dependent because beads without coupled template failed to generate transcripts (Figure 2, lanes 1 and 2). α-Amanitin at a concentration that selectively blocks plant RNA polymerase II activity (Jendrisak and Burgess, 1975; Guilfoyle and Jendrisak, 1978) inhibited transcription from washed complexes by more than 90% (lane 5).

The in vitro transcription initiation system was optimized with respect to template concentration, salt, and temperature. Transcription complexes formed between 20 and 40°C, and transcription was optimal at ~30°C (data not shown). Maximal assembly of transcription complexes on DNA beads was observed after 30-min incubation at 30°C. Transcription was approximately linear over the range of 0.25 to 1.5 pmol of template in 50-μL reaction mixes containing 150 μg of protein from whole-cell extracts (data not shown). Reproducibility between samples was high and incorporation of radiolabel into product generally varied less than 20% between replicate transcription reactions. As shown in Figure 3, transcription was

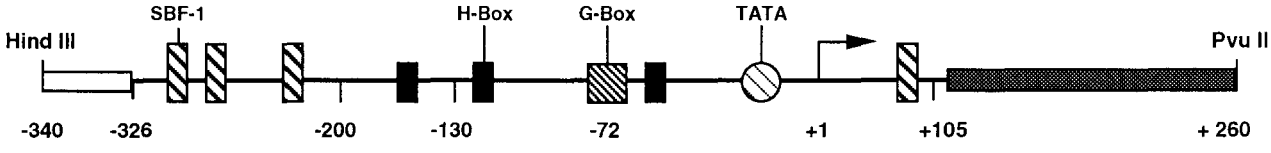

Figure 1. Organization of the CHS15 Promoter-CAT Gene Fusion Used as a Template for in Vitro Transcription.

The *cis* elements of the CHS15 promoter (−326 to +105 bp) are illustrated. The in vivo RNA start site (+1) is marked by an arrow showing the direction of transcription. A 600-bp promoter-containing fragment generated by cleavage of pCHC1 with HindIII/PvuII was used as the template for in vitro transcription experiments. This template comprises CHS15 sequences from −326 to +105 fused to the 5′ region of the CAT coding sequence. Accurate transcription initiation and full-length RNA synthesis from this template are expected to yield a 260-nucleotide transcript. Open rectangle, vector; stippled rectangle, CAT reporter gene; SBF-1, silencer binding factor-1.

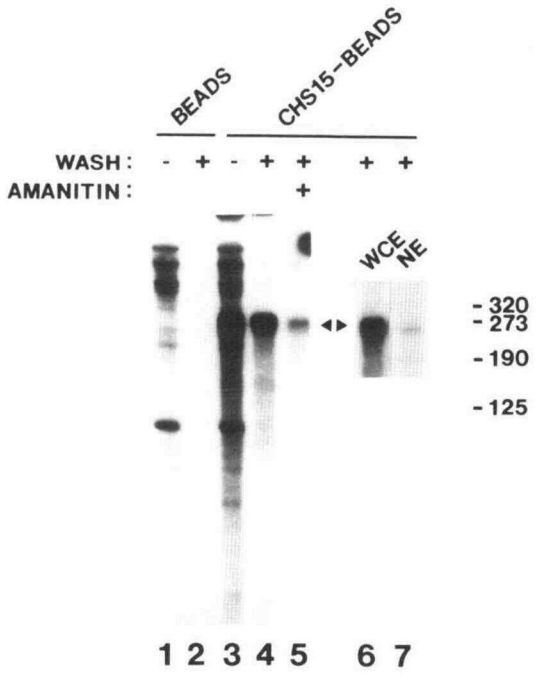

Figure 2. In Vitro Transcription with Plant Extracts.

Template dependence, wash stability, α-amanitin sensitivity, and the relative activities of whole-cell and nuclear extracts were determined using immobilized CHS15 promoter template. Each reaction contained either 150 μg of soybean whole-cell extract (lanes 1 to 6) or 25 μg of nuclear extract (lane 7), avidin-agarose beads alone (BEADS), or beads coupled to 1 pmol of CHS15 template (CHS15-BEADS). Samples were incubated at 30°C for 30 min, and either washed with 1 mL of transcription buffer to remove unbound materials (+) or left unwashed (−). To inhibit RNA polymerase II, α-amanitin was added to one reaction at 0.5 μg/mL (lane 5). All samples were processed for analysis of in vitro transcription as described in the text. DNA length markers at right are given in base pairs.

stimulated by potassium acetate (50 to 100 mM), and magnesium salts (equimolar mixtures of magnesium acetate and sulfate, 5 to 10 mM for each salt). Neither manganese chloride nor magnesium chloride could substitute for magnesium acetate/sulfate in these reactions. Maximal specific transcriptional activity per unit of protein in nuclear extracts was less (~50%) than that observed with whole-cell extracts (Figure 2, compare reaction products in lane 6 from 150 μg of whole-cell extract with those in lane 7 from 25 μg of nuclear extract), and given the relative ease of preparation, whole-cell extracts were routinely used in further experiments.

The resolution by PAGE of a single major transcription product in the reaction of the soybean whole-cell extract with the CHS15 promoter template beads suggested that CHS15 transcription was accurately initiated at the in vivo transcription start site. To confirm that transcription initiation in vitro was indeed accurate, the runoff transcripts were analyzed by primer extension, as shown in Figure 4 (PE lane). We found that the 5′ end of these transcripts mapped to the previously determined in vivo transcription start site (Ryder et al., 1987; Dron et al., 1988). In addition, CHS15 promoter templates that were 3′ truncated by an additional 145 bp gave a correspondingly smaller product in the in vitro transcription reaction (115 nucleotides compared to 260 nucleotides with the standard construct), as shown in Figure 5. Taken together, these data indicated that this in vitro transcription system provided accurate initiation from the in vivo start site followed by faithful transcription of the CHS15 promoter through to the 3′ terminus of the template.

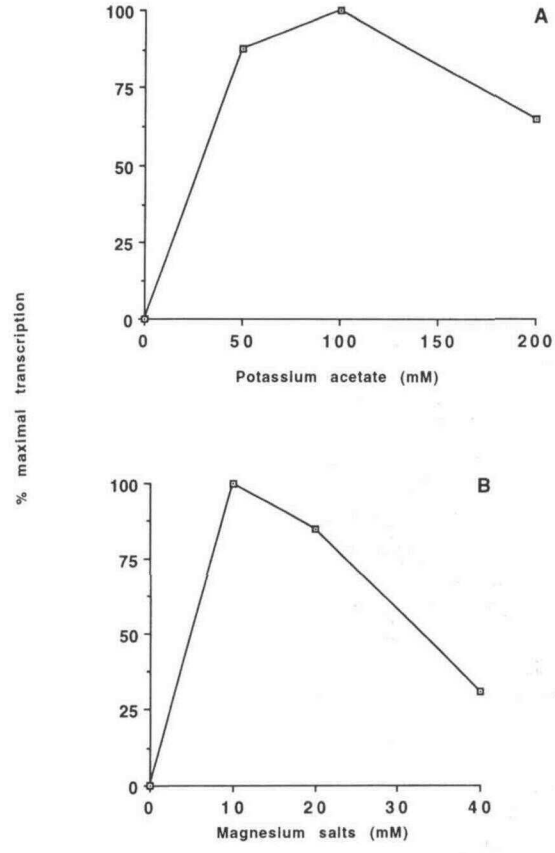

Figure 3. Optimization of Salt Concentrations for in Vitro Transcription of an Immobilized CHS15 Promoter Template by a Soybean Whole-Cell Extract.

(A) Transcription as a function of the concentration of potassium acetate.
(B) Transcription as a function of equimolar concentrations of magnesium sulfate and magnesium acetate.
Transcriptional activity was determined in standard reactions and quantified by densitometry of autoradiographs. Data are expressed as a percentage of maximum transcription.

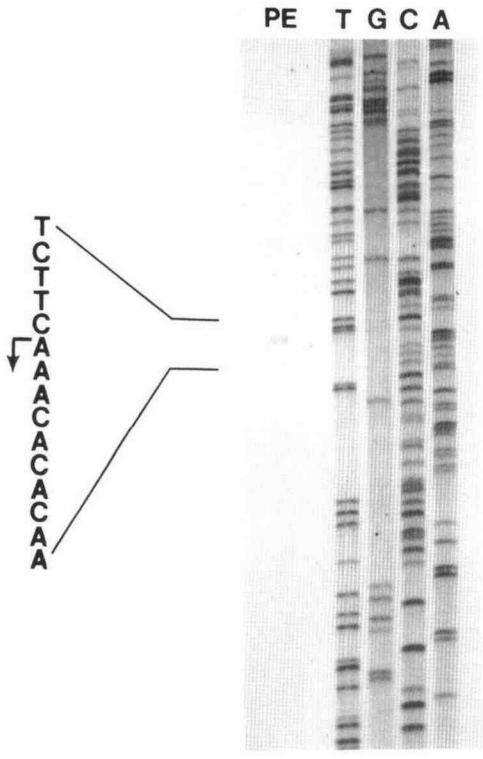

Figure 4. Primer Extension Analysis of CHS15 in Vitro Transcripts.

RNA synthesized from washed transcription complexes on CHS15 beads (0.5 pmol of template) was analyzed by primer extension. Deoxynucleotide sequencing reactions (nucleotide bases are T, thymidine; G, guanosine; C, cytosine; A, adenosine) of the CHS15 promoter and the primer extension (PE) products were fractionated on a 10% denaturing polyacrylamide gel. DNA sequences immediately adjacent to the RNA start site (arrow) of the CHS15 promoter are shown.

CHS15 Transcription by Whole-Cell Extracts from Monocot and Dicot Plants

To examine the general utility of this in vitro transcription system, whole-cell extracts from suspension-cultured cells of other plant species were prepared and tested for transcription from the CHS15 template. Soybean and bean whole-cell extracts were transcriptionally more active with this specific template than were equivalent extracts from tobacco cells or cells of the monocot rice in reactions containing similar amounts of extract protein, as shown in Figure 6. However, in all cases, transcription appeared to be accurately initiated based upon the size of the monodisperse-labeled product.

Transcription of Other Promoter Templates

A second test of the general utility of this in vitro plant transcription system was to determine whether soybean whole-cell extracts were competent to transcribe other promoter templates. Under conditions for optimal transcription of the bean CHS15 promoter, a transcription product of the appropriate size was observed with the bean CHS8 promoter (Schmid et al., 1990), as shown in Figure 7, and likewise the bean phenylalanine ammonia-lyase 2 gene promoter (Liang et al., 1989) was accurately transcribed (data not shown). Under these conditions, the rice RCH10 basic chitinase promoter (Zhu and Lamb, 1991; Zhu et al., 1993) gave a major transcription product of the appropriate size together with a second slightly smaller transcript (Figure 7). Likewise, the adenovirus 2 major-late promoter (Conaway and Conaway, 1990) generated a major transcript of the size expected for accurate initiation, together with two less prominent transcripts. The same respective product profiles were observed when the CHS8, RCH10, and adenovirus 2 major-late promoter templates were transcribed by a HeLa cell extract (Figure 7). The bean CHS8 promoter (and CHS15 promoter, data not shown) was more efficiently transcribed by the soybean cell extract optimized for CHS transcription than by the HeLa cell extract, whereas the animal virus promoter and the monocot promoter were more efficiently transcribed by the animal cell extract (Figure 7).

Dissection of the Functional Architecture of the CHS15 Promoter in Vitro

Analysis of a set of templates comprising CHS15 5′ promoter deletions showed that removal of sequences from position

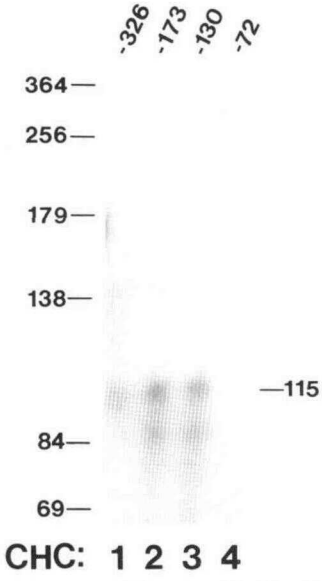

Figure 5. Transcription from 3′ Truncated CHS15 Templates Containing 5′ Promoter Deletions.

Standard in vitro transcription reactions using a soybean whole-cell extract were performed with immobilized 3′ truncated CHS15 promoter templates. The templates comprise a series of 5′ promoter deletions, with 5′ end points of: −326, CHC1; −173, CHC2; −130, CHC3; −72, CHC4. The transcription products were analyzed by PAGE and sized by reference to coelectrophoresed markers.

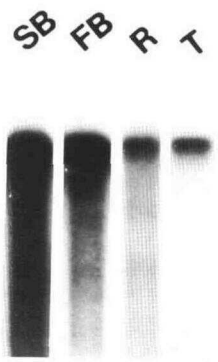

Figure 6. Transcription of the CHS15 Promoter Template with Whole-Cell Extracts from Soybean, Bean, Tobacco, and Rice Suspension-Cultured Cells.

Whole-cell extracts prepared from suspension-cultured cells of soybean (SB), bean (FB), rice (R), and tobacco (T) were incubated with CHS15 beads (0.5 pmol of template) as described in the text. Samples contained 150 μg of protein of the respective whole-cell extracts. The autoradiograph was overexpressed to reveal transcripts from the reactions with tobacco and rice extracts.

−326 to −173 increased transcription in vitro but that further deletion from −130 to −72 resulted in a severe reduction in transcription (Figure 5). The effects of these 5′ deletions on in vitro transcription closely parallel those previously observed for in vivo expression of the respective CHS15-CAT gene fusions in electroporated soybean protoplasts (Dron et al., 1988).

To delineate further details of the functional organization of the CHS15 promoter, we next examined whether extracts preincubated with DNA fragments from the CHS15 promoter region were then competent to transcribe the immobilized CHS15 template in a subsequent in vitro transcription assay, as shown in Figure 8. By using this procedure, the function of specific *cis* elements in the CHS15 promoter could be determined by assay of the effects of depletion of the cognate *trans* factors from the soybean whole-cell extract. Compared to control reactions with a pUC19 vector fragment (Figure 8, lanes 1 and 2), depletion of the soybean whole-cell extract by preincubation with CHS15 promoter fragments from position −132 to −80 (lanes 3 and 4) or from −80 to −40 (lanes 5 and 6) completely inhibited subsequent transcription from the CHS15 promoter template. In contrast, depletion of the extract by preincubation with the CHS15 promoter fragment from position −4 to +105 as a *trans* competitor had little effect on transcription (Figure 8, lanes 7 and 8) relative to the pUC19 vector control. These results indicate that accurate, efficient transcription in vitro of the CHS15 template requires interactions between specific *trans* factors in the soybean cell extract and cognate upstream *cis* elements that are proximal to the TATA box, consistent with previous in vivo functional analysis of CHS15-CAT expression (Dron et al., 1988).

The functional architecture of the CHS15 promoter was further dissected by extension of the extract depletion experiments to delineate the function of specific *cis* elements. In these experiments, *trans* competitors were either the −80 to −40 fragment of the CHS15 promoter mutated at specific sites or synthetic oligonucleotides containing specific *cis* elements (see Methods). The molar concentration of the *trans* competitor fragment in these extract depletion incubations was the same as that used to give complete inhibition of CHS15 transcription in the equivalent experiments with the wild-type −80 to −40 fragment as *trans* competitor. Prominent features of this region are a G-box sequence (CACGTG), which is found in a wide variety of plant promoters and binds a family of basic domain/leucine zipper transcription factors (Armstrong et al., 1992; Williams et al., 1992), and an H-box sequence (CCTACC), which is found in the promoters of a number of stress-inducible phenylpropanoid biosynthetic genes (Lois et al., 1989; Ohl et al., 1990).

The effects of mutations in these *cis* elements in the context of the −80 to −40 fragment of the CHS15 promoter were examined by the *trans* competitor extract depletion assay. Whereas preincubation with wild-type −80 to −40 sequences (*trans* competitor A) almost completely inhibited subsequent CHS15 transcription, preincubation with *trans* competitor A that was mutated in the G-box only inhibited transcription by ~60% compared to control reactions, as shown in Figure 9 (compare lanes 1 and 2 with lanes 5 and 6). Likewise, preincubation with

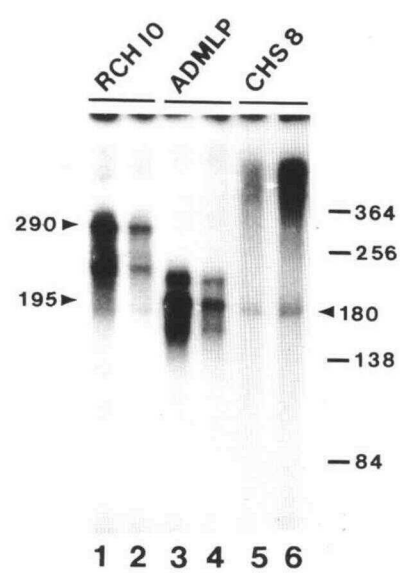

Figure 7. In Vitro Transcription of Other Immobilized Promoter Templates by a Soybean Whole-Cell Extract.

Standard in vitro transcription reactions were performed with the rice RCH10 basic chitinase promoter, adenovirus 2 major-late promoter (ADMLP), and bean chalcone synthase 8 (CHS8) promoter templates immobilized on agarose beads using HeLa (lanes 1, 3, and 5) or soybean (lanes 2, 4, and 6) whole-cell extracts prepared as described in the text. Transcription products were analyzed by PAGE and sized by comparison with coelectrophoresed standards.

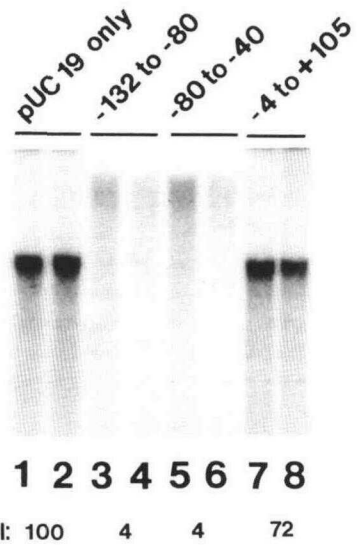

Figure 8. Mapping Functional Domains of the CHS15 Promoter.

Samples containing 135 μg of protein of soybean whole-cell extract were incubated with 5 pmol of nonspecific DNA competitor (a 181-bp fragment of HindIII- and PvuII-digested pUC19) or 5 pmol of the specific *trans* competitor containing CHS15 promoter sequences as indicated. Following incubation at 30°C for 30 min, 0.5 pmol of CHS15 template on beads was added to each reaction. Incubation was continued for another 30 min. The samples were then washed and processed for analysis of in vitro transcription as described in the text. Transcription products were quantified by densitometry and the mean of duplicate samples expressed as a percentage of the positive control reaction (lanes 1 and 2).

trans competitor A mutated in the H-box also inhibited subsequent transcription from the CHS15 promoter template by ~60% (Figure 9, lanes 7 and 8). Thus, the effects of specific mutations in the G-box and H-box *cis* elements in the context of the −80 to −40 sequence indicate that binding of the respective cognate *trans* factors to the G-box and H-box sequences together can largely account for the total inhibition of CHS15 transcription resulting from depletion of the soybean extract by preincubation with the wild-type −80 to −40 sequences in *trans*. Consistent with this conclusion, an oligonucleotide containing CHS15 sequences located from −56 to −40 downstream of the G-box and H-box *cis* elements (see Methods) inhibited transcription by only 16% (Figure 9, lanes 13 and 14).

The hypothesis that G-box and H-box *cis* elements have important roles in activating transcription from the CHS15 template in vitro was further supported by extract depletion experiments with oligonucleotides containing individual G-box or H-box *cis* elements, as shown in Figures 9 and 10. Competition with either the G-box or H-box *cis* element in *trans* inhibited transcription by 45 to 60% compared to control reactions (Figure 9, lanes 1 and 2 compared with lanes 9 to 12; Figure 10, lane 1 compared with lanes 2 and 3), whereas transcription following extract depletion by both *cis* elements in *trans* was reduced by ~95% relative to the level in positive control reactions (Figure 10, lane 4). These results confirmed that the G-box and the H-box *cis* elements make major contributions to the transcription of the CHS15 promoter in vitro, and indicated that both *cis* elements in combination are required for maximal activity.

DISCUSSION

We report here the development of an accurate, efficient in vitro system in which soybean whole-cell extracts and nuclear extracts direct transcription from an immobilized template. This

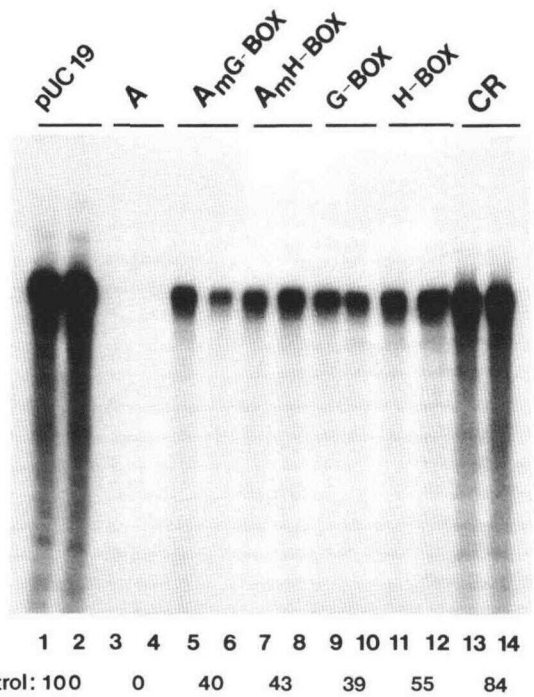

Figure 9. Functional Analysis of the CHS15 Promoter in Vitro by Selective Depletion of Soybean Extracts with Specific *trans* Competitor Sequences.

Whole-cell extracts (135 μg) were incubated with 5 pmol of a nonspecific *trans* competitor (the 181-bp pUC19 fragment) or with 5 pmol of a specific *trans* competitor as indicated. *trans* competitor A is the CHS15 −80 to −40 promoter fragment; A_mG-box is *trans* competitor A mutated in the G-box; A_mH-box is *trans* competitor A mutated in the H-box; CR is the CHS15 −56 to −40 sequence fragment (for sequences of these oligonucleotides see Methods). After incubation at 30°C for 30 min, 0.5 pmol of CHS15 DNA template on beads was added and the reaction continued for an additional 30 min. Immobilized complexes were then washed and processed for analysis of in vitro transcription as described in the text. Transcription products were quantified by densitometry and the mean of duplicate samples expressed as a percentage of the positive control reaction (lanes 1 and 2).

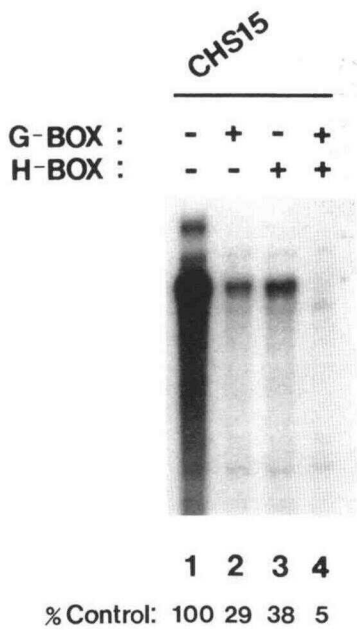

Figure 10. G-Box and H-Box *cis* Elements Are Both Essential for Maximal Transcription of a CHS15 Promoter Template in Vitro.

Reactions contained soybean whole-cell extracts (150 μg) and either 5 pmol of a nonspecific *trans* competitor, the pUC19 181-bp DNA fragment, or 5 pmol of the specific *trans* competitor oligonucleotides indicated (see Methods for oligonucleotide sequences). Reactions were incubated at 30°C for 30 min, 0.5 pmol of CHS15 DNA coupled to beads was then added, and the incubation was continued for an additional 30 min. Immobilized transcription complexes were then washed and reprocessed for analysis of in vitro transcription as described in the text. Transcription products were quantified by densitometry and the data are expressed as a percentage of the positive control reaction (lane 1).

(+), with prior *trans* competition; (−), without prior *trans* competition.

novel, homologous plant in vitro transcription initiation system was exploited to examine the functional organization of the bean CHS15 promoter, and we demonstrated that the transcription of this defense gene is critically dependent on *trans* factors in the soybean whole-cell extract that interact with two small regions of the promoter containing G-box (CACGTG) and H-box (CCTACC) *cis* elements, respectively.

In studies on animal and yeast gene expression, the molecular basis of promoter-specific transcription by RNA polymerase II has been elucidated largely through in vitro studies in which whole-cell or nuclear extracts direct transcription from cloned promoter templates. In contrast, such approaches have not been possible in comparable studies of plant gene regulation mechanisms because of the lack of simple, widely applicable procedures for the preparation of transcriptionally competent cell extracts from physiologically relevant plant systems. Transcription initiation has been observed with wheat germ extracts, but only after substantial fractionation, possibly because of the presence of inhibitors in crude extracts. Recently, extracts of isolated wheat germ chromatin have been reported to give efficient, and, on the basis of product size, accurate transcription of templates containing the TC7 promoter of the Agrobacterium Ti plasmid (Yamazaki et al., 1990a) or a synthetic plant promoter (Yamazaki et al., 1990b). In the latter case, transcription is stimulated by addition of the tobacco TAF-1 *trans*-acting factor that binds to a cauliflower mosaic virus 35S transcript *cis* element present in the synthetic promoter template (Yamazaki et al., 1990b).

A common characteristic of yeast and the initial animal in vitro transcription systems is that they were obtained from homogeneous, relatively undifferentiated, rapidly growing cell cultures. In contrast, wheat germ is a highly specialized, essentially dormant tissue, and hence may be deficient in transcription factors required for specific gene regulation mechanisms. By analogy with the yeast and animal systems, we reasoned that suspension-cultured plant cells derived from callus might be an appropriate source for the preparation of transcriptionally active extracts. Moreover, cell suspension cultures exhibit selective regulation of gene expression patterns in response to developmental and environmental cues including hormones, light, and fungal elicitors, and these responses mimic well-characterized physiological processes in intact plant tissues (Hahlbrock and Scheel, 1989; Lamb et al., 1989).

By the modification of methods used for the preparation of active extracts from human and yeast cells (Manley et al., 1980; Lue and Kornberg, 1987; Woontner and Jaehning, 1990), whole-cell and nuclear extracts from suspension-cultured plant cells were obtained that accurately and efficiently transcribed a bean CHS15 gene promoter as template. A major problem was the appearance of radiolabeled products that did not arise from template-dependent RNA synthesis and that hindered the identification and quantification of the product of the template-dependent transcription reactions. Background radiolabeling has also been observed in previous studies with crude whole-cell extracts from animal and plant cells (Manley et al., 1980; Flynn et al., 1987; Cooke and Penon, 1990; Roberts and Okita, 1991). Protoplasts from plant suspension-cultured cells have been shown to be suitable material for preparing transcriptionally active nuclear extracts (Roberts and Okita, 1991). However, background radiolabeling is very high with these extracts, and this necessitated the use of specially constructed templates to generate RNA with polyadenylate tails such that this particular subset of transcription products could then be recovered from the crude reaction mixture by semiquantitative batch hybridization. In contrast, the immobilized transcription system described in our study permits the efficient isolation of functional transcription complexes from either nuclear or whole-cell extracts. This approach allows the facile washing of assembled transcription complexes to remove material that generates background labeling in unwashed incubations prior to the introduction of ribonucleoside triphosphates to initiate template-dependent RNA synthesis, resulting in the almost complete recovery of transcriptional activity on the immobilized template in the absence of background labeling.

In vitro transcription reactions using this immobilized template system were successful with both whole-cell extracts and nuclear extracts of suspension-cultured soybean cells. Transcription was catalyzed by RNA polymerase II and was accurate with respect to the in vivo initiation site for CHS15, as demonstrated by the appropriate size of the single monodisperse product, primer extension analysis, and generation of an appropriately sized shorter transcript from a 3′ truncated template. As previously noted with yeast extracts (Woontner and Jaehning, 1990; Woontner et al., 1991), the specific transcriptional activity per unit of extract protein was slightly greater in soybean whole-cell extracts than in nuclear extracts. The soybean whole-cell extract also transcribed several other immobilized templates including the bean CHS8 promoter, the rice RCH10 chitinase promoter, and the adenovirus 2 major-late promoter. However, we have not attempted to optimize conditions for the transcription of templates other than the bean CHS15 promoter. Active extracts were obtained from suspension-cultured cells of other species including the monocot rice. Interestingly, the extracts from the nonlegume species were two- to threefold less active than the homologous extracts in transcribing the legume gene promoter template. This might reflect differences between plant species in *trans* factors required for transcription of the bean CHS15 promoter, or the procedures developed for the preparation of active soybean extracts might not be optimal for nonlegume species. However, accurate initiation of transcription from the CHS15 promoter was observed in the tobacco and rice whole-cell extracts, and we anticipate that transcription from immobilized templates by extracts prepared by the simple procedures described here will find widespread use in the investigation of plant gene regulation mechanisms and the functional properties of isolated *trans* factors.

Previous studies of the CHS15 promoter have shown that a silencer is located between positions −326 and −173, and that sequences between positions −130 and +104 are sufficient for elicitor-regulated expression in electroporated soybean protoplasts (Dron et al., 1988). In vitro analysis of the corresponding set of 5′ deletions revealed a similar pattern, such that deletion from −130 to −72 abolished CHS15 transcription by the soybean whole-cell extract. We further examined the functional organization of this proximal region of the CHS15 promoter by experiments in which transcription from the immobilized template was assayed in vitro following selective depletion of the soybean whole-cell extract by prior incubation with specific CHS15 promoter sequences. A similar approach has been used to examine functionally conserved regions of different promoters (Hai et al., 1988; Andrisani et al., 1989), and recently the organization of the c-*fos* promoter was delineated in vitro by a combination of functional assays of mutated promoters and extract depletion by *trans* competition with the serum response element and other *cis* elements (Hipskind and Nordheim, 1991).

In our experiments, preincubation of the soybean whole-cell extract with CHS15 sequences from −4 to +105 had little effect on subsequent transcription of the immobilized CHS15 promoter compared to positive controls with equivalent extracts that had been incubated with unrelated pUC19 vector sequences. Thus, this region of the promoter does not contain *cis* elements that bind *trans* factors in the soybean extract essential for CHS15 transcription. In contrast, depletion of the extract by preincubation with CHS15 sequences from either positions −132 to −80 or from positions −80 to −40 as *trans* competitors essentially abolished activity, indicating that these specific regions contain *cis* elements that bind *trans* factors in the soybean whole-cell extract that are essential for transcription from the CHS15 promoter in vitro. This conclusion is consistent with previous observations that 5′ deletion from position −130 to −72 abolishes elicitor-induced expression of a CHS15-CAT gene fusion in electroporated protoplasts derived from suspension-cultured soybean cells (Dron et al., 1988).

It is apparent from analysis of the transcription of 5′ promoter deletions by the soybean whole-cell extract and the transcription of the full promoter by extracts depleted by prior *trans* competition with promoter fragments that the minimal promoter containing a functional TATA box is not sufficient to drive CHS15 transcription in the absence of interactions between upstream *cis* elements and their cognate *trans* factors. These observations are in accord with the prior in vivo analysis of the functional architecture of the CHS15 promoter (Dron et al., 1988) and are similar to analyses of a number of animal promoters, such as the in vitro transcription of the mouse albumin promoter, which depends on upstream components in addition to a functional TATA box (Gorski et al., 1986).

Our data indicate that within the TATA-proximal region of the CHS15 promoter, *trans* factors that bind to two short sequences centered on the G-box and H-box *cis* elements, respectively, make major contributions to the in vitro transcription of the promoter, such that simultaneous depletion of these factors from the soybean whole-cell extracts abolishes CHS15 transcription and both sets of factors are required for maximal activity in vitro. Both *trans* competitor A mutated in the G-box and *trans* competitor A mutated in the H-box contain mutations at specific residues that have been shown to be important for the respective functions of these *cis* elements in vivo (Lam et al., 1989; Marcotte et al., 1989; Block et al., 1990; DeLisle and Ferl, 1990; Donald and Cashmore, 1990; Loake et al., 1992; O. Faktor, J. Kooter, R. A. Dixon, and C. J. Lamb, unpublished data) and binding of the cognate *trans* factors in vitro (Armstrong et al., 1992; Williams et al., 1992; Yu et al., 1993; A. Levine, J. A. Arias, and C. J. Lamb, unpublished data). In addition, the synthetic *cis* elements used in the subsequent depletion experiment contain only short stretches of wild-type flanking sequence (5 bp 5′ and 0 bp 3′ of the H-box CCTACC core, 6 bp 5′ and 5 bp 3′ of the G-box CACGTG core; see Methods). Hence, we conclude that in vitro transcription of the CHS15 promoter is critically dependent on the interaction between the G-box and H-box *cis* elements and their cognate *trans* factors in the soybean whole-cell extract. Such interactions may also account, at least in part, for the inhibition of CHS15 transcription by extract depletion with the −132 to −80 fragment as a *trans*

competitor because this region contains an H-box in the inverted orientation (−121 to −126) and the ACGT core (−96 to −93) of the G-box, although we cannot rule out the possibility that interactions between other *cis* elements and their cognate *trans* factors in the soybean whole-cell extract might also contribute to CHS15 transcription.

The properties of the CHS15 promoter deduced from these in vitro transcription experiments are in accord with the emerging in vivo functional analysis of CHS15 expression, which shows that both the G-box and H-box *cis* elements are essential for cell type–specific expression in the inner epidermis of the corolla during flower development, stress induction in wounded tissue, or during a hypersensitive resistance response (O. Faktor, J. Kooter, R. A. Dixon, and C. J. Lamb, unpublished data), and modulation by the phenylpropanoid central pathway intermediate *p*-coumaric acid (Loake et al., 1992). Whereas the G-box is found in a wide range of inducible plant promoters, the H-box appears to have a more limited distribution (Williams et al., 1992; Yu et al., 1993), and the combinatorial specificity conferred by the interaction between the G-box and H-box *cis* elements in the CHS15 promoter and the promoters of genes encoding other phenylpropanoid biosynthetic enzymes (Lois et al., 1989; Ohl et al., 1990; Leyva et al., 1992) establishes a unique pattern of promoter activity not observed with promoters containing only one of these two elements, e.g., ribulose bisphosphate carboxylase small subunit (*rbcS*) and *Em* from wheat (Marcotte et al., 1989; Williams et al., 1992). The ability to study such functional interactions in vitro will facilitate the dissection of the structural and mechanistic basis for combinatorial specificity in plant gene regulation. Moreover, the close correspondence between the functional architecture of the CHS15 promoter in vivo and that delineated here by analysis of transcription in vitro indicates that the in vitro transcription system retains key physiological attributes and, hence, may be useful for studying the molecular events involved in the terminal stages of signal pathways for gene regulation by fungal elicitors and other environmental stimuli or developmental cues.

METHODS

Plant Cell Cultures

Soybean (*Glycine max*), bean (*Phaseolus vulgaris*), and tobacco (*Nicotiana tabacum*) cell suspension cultures were established and maintained as previously described (Cramer et al., 1985; Hahn et al., 1987; Dron et al., 1988). Suspension cultures of rice (*Oryza sativa*) cells were a generous gift of Thomas Hodges (Purdue University, West Lafayette, IN) and were maintained on N7 medium (Zhu and Lamb, 1991). Suspension cultures from each line were transferred to fresh media at weekly intervals and shaken at 120 rpm in the dark at 26 to 28°C.

Buffers

For the preparation of nuclear extracts, the buffers are as follows. Isolation buffer, 25 mM Tris-HCl, pH 8.0, 2.5% (w/v) Ficoll 400, 5.0% (w/v) dextran T-40, 5 mM EDTA, 0.44 M sucrose, 2 mM spermine, 1 mM NaF, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5% (v/v) Triton X-100; extraction buffer, 50 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.2 mM EGTA, 5% (v/v) glycerol (ultrapure; U.S. Biochemical), 0.48 M KCl, 1 mM NaF, 20 mM 2-mercaptoethanol, 0.1 mM PMSF, 2 μg/mL leupeptin, 3 μg/mL pepstatin A, and 40 μg/mL antipain; dialysis buffer, 25 mM Hepes-KOH, pH 8.0, 20% (v/v) glycerol, 0.1 mM EDTA, 50 mM KCl, 0.5 mM DTT, and 0.1 mM PMSF.

For the preparation of whole-cell extracts, the following buffers were used. The homogenization buffer contained 200 mM Tris base, 390 mM ultrapure $(NH_4)_2SO_4$ (Pharmacia, Sweden), 10 mM $MgSO_4$, 20% (v/v) glycerol, 5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine. The pH was brought to 7.9 with glacial acetic acid, 1.5% (w/v) polyvinylpolypyrrolidine was added, and the mixture was then stirred overnight at 4°C. Immediately prior to use, 5 mM DTT, 1 mM PMSF, 0.5 μg/mL leupeptin, 2.5 μg/mL antipain, 0.35 μg/mL bestatin, and 0.4 μg/mL pepstatin A (all protease inhibitors were from Sigma) were added. The resuspension buffer was the same as the dialysis buffer, except that it contained 10 mM PMSF, 5 μg/mL leupeptin, 25 μg/mL antipain, 3.5 μg/mL bestatin, and 4 μg/mL pepstatin A. The dialysis buffer contained 50 mM Hepes-KOH, pH 7.9, 20% (v/v) glycerol, 10 mM EGTA, and 10 mM $MgSO_4$, with 5 mM DTT and 1 mM PMSF added just before use.

Nuclear Extracts

Soybean suspension cultures were collected on Miracloth (Calbiochem) by vacuum filtration. Approximately 50 g (fresh weight) of cells was divided into 10-g portions and frozen in liquid N_2. Frozen cells were pulverized to a fine powder using a mortar and pestle on dry ice. All subsequent steps were at 4°C. Pulverized cells were extracted by stirring for 5 min in isolation buffer (10 mL of buffer per g of cells). The slurry was sequentially filtered through two layers of cheesecloth with 80- and 42-μm nylon meshes, respectively. Nuclei in the filtrate were collected by centrifugation at 2500*g* for 5 min, resuspended in 250 mL of isolation buffer, and collected by centrifugation. This wash step was then repeated with isolation buffer lacking Triton X-100. Nuclei were resuspended in 10 mL of isolation buffer, centrifuged at 2500*g* for 10 min, and resuspended in exactly two volumes of extraction buffer. This suspension was then incubated on a rotary mixer for 30 min to extract nuclear proteins. The sample was centrifuged at 100,000*g* for 1 hr, and the upper two-thirds volume of the supernatant was transferred to dialysis tubing. Samples were dialyzed twice against 1 L of dialysis buffer for 4 hr. Finally, the dialyzate was centrifuged at 5000*g* to pellet insoluble material, and the remaining supernatant was aliquoted, frozen in liquid N_2, and stored at −80°C for up to 2 months. Protein concentrations in the final preparations were typically 200 to 300 μg/mL.

Whole-Cell Extracts

Suspension-cultured cells were harvested in late-log phase by vacuum filtration on Miracloth, and then washed with ice-cold deionized H_2O and 200 mL of ice-cold homogenization buffer, respectively. All subsequent steps were performed at 4°C. Cells (100 g fresh weight) were resuspended in 200 mL of ice-cold homogenization buffer and placed into an ice-cooled, 350-mL stainless steel chamber of a Bead Beater homogenization apparatus containing 100 g of glass beads (for rice, 0.2- to 0.3-mm diameter; for all other plant cells tested, 0.5-mm diameter). Cells were lysed by 30-sec grinding and rest cycles (8 to 12 cycles) until at least 90% of the population was estimated to be broken. The

homogenate was centrifuged at 5000*g* for 5 min, and the supernatant was transferred to a 500-mL beaker containing a magnetic stir bar. While stirring, the solution was brought to 0.9 M $(NH_4)_2SO_4$ by the dropwise addition of a 4 M $(NH_4)_2SO_4$ solution brought to pH 7 with H_2SO_4. The homogenate was stirred for another 30 min and then centrifuged at 110,000*g* for 2 hr at 0°C. The upper, white (lipid) layer was carefully removed, and the remaining supernatant was transferred to a 200-mL beaker and stirred. Solid ultrapure $(NH_4)_2SO_4$ (0.35 g/mL homogenate) and a solution of 1 M KOH (10 μL/mL $(NH_4)_2SO_4$) were added over a 1-hr period while stirring. After 30 min, the samples were centrifuged at 110,000*g* for 30 min at 0°C. The supernatant was then decanted and the pellet was gently resuspended in resuspension buffer (45 μL/g of suspension culture), placed in Spectra/Por No. 2 dialysis membrane, and dialyzed twice against 1 L of dialysis buffer overnight. The dialyzate was collected and centrifuged at 5000*g* to pellet insoluble material. Aliquots were frozen in liquid N_2 and stored at −80°C for up to 2 months. Protein concentrations in the final whole-cell extract were typically 20 to 40 mg/mL.

HeLa Cell Extracts

HeLa whole-cell extracts were prepared as previously described (Arias and Dynan, 1989).

DNA Templates

pCHC1 is a pUC19 plasmid containing CHS15 sequences from position −326 to +105 upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (Dron et al., 1988). Plasmid DNA was produced in the DH5α strain of *Escherichia coli*, isolated by alkaline lysis, and purified with a CsCl gradient (Sambrook et al., 1989). The supercoiled form of the pCHC1 plasmid was isolated and used to make template.

The pCHC plasmids, which contain a set of 5′ nested CHS15 promoter deletions (Dron et al., 1988), were digested with HindIII and BamHI to give fragments of 450 (CHC1), 300 (CHC2), 260 (CHC3), and 200 bp (CHC4). Digestion of these plasmids with HindIII and BamHI results in 3′ truncated templates, compared with the standard CHS15 template obtained by digestion of pCHC1 with HindIII and PvuII (see above), leading to an expected transcript of 115 nucleotides. The CHS8 promoter template was generated by digestion of pCHS8 (Schmid et al., 1990) with EcoRI and HindIII. This template contains CHS8 promoter sequences to −1009 and is expected to generate a transcript of 180 nucleotides. The adenovirus 2 major-late promoter template was generated from a 60-bp fragment (position −50 to +10) cloned into pUC18 (Conaway and Conaway, 1990). Digestion with EcoRI and BglI gives a promoter-containing fragment of 250 bp, with an expected transcript size of 195 nucleotides. For preparation of the RCH10 chitinase template, sequences from −74 to +116 (Zhu et al., 1993) were cloned in pUC19. The template fragment was isolated by EcoRI digestion and is expected to give a 290 nucleotide transcript.

Templates were prepared and coupled to avidin-agarose beads essentially as previously described by Arias and Dynan (1989). Template DNA digested with either EcoRI or HindIII (see above) was extracted with phenol/chloroform (1:1), collected by ethanol precipitation, and incubated with the DNA polymerase I large fragment (Klenow enzyme), dCTP, dGTP, dTTP, and biotin-7-dATP (Bethesda Research Laboratories). The resulting biotinylated DNA was extracted with phenol/chloroform (1:1), collected by ethanol precipitation, and digested with a second restriction enzyme. The resulting biotinylated template was fractionated by PAGE and recovered by electroelution. Biotinylated template was then coupled overnight at 4°C to avidin-agarose beads (Pierce Chemical Co.) and washed with 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA (TE buffer) to remove unbound template. The concentration of template immobilized on the agarose beads was calculated as described previously (Arias and Dynan, 1989) and was typically 60 to 100 pmol of DNA per mL of avidin-agarose beads. Immobilized templates (DNA-Beads) were stored at 4°C in an equal volume of TE buffer containing 0.1% (w/v) sodium azide for up to 3 months.

Oligonucleotides

The 181-bp pUC19 fragment and the 119-bp CHS15 −4 to +105 fragment were obtained from the pUC19 and pCHC1 plasmids, respectively, after HindIII and PvuII digestion, native gel fractionation, and DNA electroelution by standard methods (Sambrook et al., 1989). Other *trans* competitor DNA was made on a Cyclone Plus DNA synthesizer (Millipore, Bedford, MA). Complementary strands of synthetic oligonucleotides were annealed as described by Yu et al. (1993). A double-stranded H-box oligonucleotide was kindly provided by Lloyd Yu (Noble Foundation). Synthetic oligonucleotides used are shown below, with putative *cis* elements shown in boldface and mutated sequences in italics:

−132 to −80 bp
5′-AGACAT**GGTAGG**CAGTGCAAAAAATATATGTTTTCAACGTAAGAAGG-CTTTGG-3′;

trans competitor A (−80 to −40 bp)
5′-GTGTTG**CACGTG**ATACTCA**CCTACC**CTACTTCCTATCCA-3′

trans competitor A mutated in the H-box
5′-GTGTTG**CACGTG**ATACTCA***CAGTCT****GA*ACTTCAGTTCCA-3′

trans competitor A mutated in the G-box
5′-GTGTTG***GCTCTG***ATACTCA**CCTACC**CTACTTCCTATCCA-3′

H-box
5′-CGACTCA**CCTACC**TGACATGCTACGCAG-3′

G-box
5′-GGTGTTG**CACGTG**ATACTGCA-3′

−56 to −40 sequence
5′-CCCTACTTCCTATCCA-3′

In Vitro Transcription

Immobilized templates (0.5 to 1 pmol) were incubated in 50-μL reactions containing either nuclear or whole-cell extracts (see legends to Figures 2, 8, 9, and 10 for concentrations), transcription buffer (50 mM Hepes-KOH, pH 8.0, 5% [v/v] glycerol, 50 mM potassium acetate, 5 mM magnesium acetate, 5 mM magnesium sulfate, 2.5 mM DTT, 5 mM EGTA), plus 0.5 units of Inhibit-Ace (5′ Prime–3′ Prime Inc., Boulder, CO), and 1 unit of RNase Block II (Stratagene) for 30 min at 30°C. To partially purify the resultant immobilized transcription complexes, 1 mL of ice-cold transcription buffer was added to the reactions, the tubes were gently inverted, and the DNA beads were pelleted in a microcentrifuge (Beckman) for 10 sec at room temperature. The supernatant was then carefully and completely removed. DNA-bead pellets were then resuspended in transcription buffer containing RNase inhibitors as given above. To this mixture was added 500 μM ATP, CTP, and GTP, 20 μM UTP, and 10 μCi of α-^{32}P-UTP (800 Ci/mmol) to label

RNA transcripts in a final volume of 50 μL. Incubation was for 30 min at 30°C. Samples were then extracted and processed for polyacrylamide fractionation as described by Arias and Dynan (1989). Denaturing polyacrylamide gels (6 to 8%) were used to fractionate RNA products (Arias and Dynan, 1989). Following electrophoresis, gels were fixed for 20 min in 5% (v/v) methanol and glacial acetic acid, and then dried by heating in vacuo. Radiolabeled products were detected by autoradiography with XAR-film (Kodak). Quantitative comparisons of bands on autoradiographs of individual gels were made by densitometry (LKB II Ultrascan).

Extract Depletion by *trans* Competition

In these experiments, 5 pmol of double-stranded oligonucleotide (see above) or DNA fragment was preincubated with soybean whole-cell extract for 30 min at 30°C. To these samples was then added 0.5 to 1.0 pmol CHS15 promoter template on beads, followed by a second incubation at 30°C for 30 min. These immobilized transcription complexes were then washed and analyzed for transcription activity under the standard conditions described above.

Primer Extension and Nucleotide Sequencing

Unlabeled RNA products from transcription reactions were analyzed by primer extension (Kadonaga, 1990) with a gel-purified CHS15 template primer (5′-GGTTAAAAGATGAAAGAAGTGACCAAGTTCAC-AGC-3′) labeled with polynucleotide kinase and γ-^{32}P-ATP. Dideoxy sequencing reactions (Sanger et al., 1977) were performed with the CHS15 template using a T7 sequencing kit (Pharmacia), Sequenase enzyme (U.S. Biochemicals), and the CHS15 primer. Labeled products from both primer extension and nucleotide sequencing reactions were fractionated on denaturing 10% polyacrylamide gels and identified by autoradiography as previously described.

ACKNOWLEDGMENTS

We thank Cindy Doane for help in preparing the manuscript, Jim Kadonaga (University of California, San Diego) for helpful discussions, Tom Hodges (Purdue University, West Lafayette, IN) for rice cultures, and Ouriel Faktor (Salk Institute) and Lloyd Yu (Noble Foundation) for providing plasmids and oligonucleotides. This research was supported by grants to C.J.L. from the U.S. Department of Agriculture (91-037303-6422) and the Samuel Roberts Noble Foundation. J.A.A. thanks the National Science Foundation for a Minority Postdoctoral Research Fellowship (DMB-9006142).

Received November 16, 1992; accepted February 17, 1993.

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