THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 31, Issue of November 5, pp. 23652-23660, 1993
Printed in U.S.A.

Hormone Response Complex in a Novel Abscisic Acid and Cycloheximide-inducible Barley Gene*

(Received for publication, May 3, 1993, and in revised form, July 12, 1993)

Qingxi Shen, Scott J. Uknes‡, and Tuan-Hua David Ho§

From the Plant Biology Program, Department of Biology, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63130

The phytohormone, abscisic acid (ABA), plays a variety of roles during seed development and in the plant's response to environmental stresses. To study the molecular action of ABA, we have isolated a single copy ABA-induced gene, *HVA22*, which is mapped to barley chromosome 1. The *HVA22* gene can be induced by either ABA or the protein synthesis inhibitor, cycloheximide, and addition of both inducers to barley aleurone layers has a synergistic effect on the expression of this gene. Sequence comparison indicates that the *HVA22* gene product is highly homologous to the product of human *DP1* gene, which is likely to contribute to colorectal tumorigenesis. The hormonal regulation of *HVA22* expression has been studied, and there appear to be at least three elements, two located in the promoter and one in the first intron, which are essential for the high level of ABA induction of HVA22 expression. Among the promoter elements is a homolog of ABA response element, which has been shown to be important in the expression of other ABA-induced genes in plants. We suggest that the barley *HVA22* gene product is likely a regulatory protein, and the ABA induction of this gene requires the action of a complex set of hormone response elements.

The phytohormone, abscisic acid (ABA),[1] is well known to mediate various development and physiological processes, including stomatal function, seed development, and the plant's response to drought, salinity, and cold stress (1, 2). During seed development, ABA is believed to play an important role in embryogenesis, storage protein synthesis, desiccation tolerance, and the onset and maintenance of dormancy (for review, see Ref. 3). Two peaks of ABA levels are observed during the seed development of barley (4), wheat (5), and *Arabidopsis* (6). *Late embryogenesis abundant* (*Lea*) genes, whose developmental expression coincides with the rise in endogenous ABA in developing seeds, have been described in various species including wheat, rice, barley, rape, and carrot (7, 8). These *Lea* genes have been grouped according to the homology in their deduced amino acid sequences (7). The expression of some *Lea* genes is closely correlated with the development of desiccation tolerance in embryos (9). It is hypothesized that cellular proteins are stabilized during desiccation via interactions with Lea proteins (7). During postgermination growth, gibberellin (GA), another phytohormone, induces the expression of genes necessary for the utilization of stored seed reserves and for seedling growth. Abscisic acid at this stage antagonizes the effect of GA. For example, GA enhances, while ABA inhibits, the synthesis of hydrolases (such as α-amylase) in barley aleurone layers (10). The ABA inhibition appears to be at both the transcriptional and post-transcriptional level (10, 11).

In vegetative tissues, ABA level increases in response to drought, salt, and cold stress (12–14). The increase in ABA level induces the expression of *Rab* (*response to ABA*) genes in rice (15) and maize (16). Some RAB proteins have a positively charged domain, which was suggested to bind nucleic acids (15). Another gene induced by ABA and water stress encodes a protein containing the consensus sequence of RNA-binding protein (17). Recently cloned from barley was an ABA- and GA-modulated gene encoding an aldose reductase, which is involved in the synthesis of sorbitol (18). Because sorbitol is a common cell osmolyte in animal cells, and probably also in plant cells, this result further indicates the importance of ABA in the protection of cells under drought stress. Involvement of ABA in cold acclimation was reported in plants of *Solanum* (12), *Nicotiana tabacum* (19), and in cultured cells of winter rape (*Brassica napus*) (20), winter wheat, and rye (21).

In barley aleurone layers, ABA induces more than a dozen polypeptides (22). Most of the proteins are heat stable (23). A 36-kDa protein has some common antigenic determinants with a barley lectin specific for glucosamine, mannosamine, and galactosamine (22). A 21-kDa protein is an α-amylase/subtilisin inhibitor, which is synthesized in the endosperm during seed development, and possibly serves to protect seeds from pathogens and/or to maintain seed dormancy (24, 25).

Although the physiological roles of ABA have been well studied, the molecular mechanism of ABA action is still poorly understood. Efforts are being made to identify the ABA receptors, to delineate ABA response elements, and to isolate trans-acting factors binding to the cis-acting elements. High affinity binding sites for ABA have been found on the plasmalemma of *Vicia faba* guard cells (26). Mundy *et al.* (27) reported that the sequence element between −294 and −52 of rice *Rab-16A* gene was sufficient to confer ABA-dependent expression of the chloramphenicol acetyltransferase reporter gene in rice protoplasts. They also identified the nuclear factor(s) binding to the conserved sequence motif I

* This work was supported by grants from the Nationl Science Foundation and the United States Department of Agriculture (to T.-H. D. H.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L19119.

‡ Present address: Ciba-Geigy, Inc., 3054 Cornwallis Rd., P. O. Box 12257, Research Triangle Park, NC 27709-2257.

§ To whom correspondence should be addressed.

[1] The abbreviations used are: ABA, abscisic acid; ABRE, ABA response element; Lea, *late embryogenesis abundant*; GA, gibberellin; Rab, response to ABA; bp, base pair(s); OP-G box, G box in an opposite orientation; GUS, β-glucuronidase.

(TACGTGGC) and motif II (IIa: CGCCGCGCCTGC; IIb: CGC/GCGCGCT). Marcotte *et al.* (28) have identified a 260-bp fragment (−168 to +92) of wheat *Em* gene that can lead to a 15–20-fold induction by ABA in β-glucuronidase (GUS) expression. Recently, Guiltinan *et al.* (29) reported the cDNA cloning of a plant leucine zipper protein (EmBP-1) that binds to the 8-bp sequence (CACGTGGC) in a 75-bp fragment of *Em* gene.

To further elucidate the mode of ABA action in regulating gene expression, we isolated several cDNA clones corresponding to ABA-induced proteins in barley aleurone layers. Here we report a novel member among these, designated HVA22. The expression of HVA22 is rapidly induced by ABA and cycloheximide. It codes for a 15-kDa protein with several interesting features, and its sequence is homologous to human *DP1* gene, which is involved in colorectal tumorigenesis. Analyses of cis-acting regulatory elements of *HVA22* gene by transient assay with *GUS* as the reporter gene enabled us to identify at least three elements, including the first intron, to be necessary for ABA induction of HVA22 in barley aleurone layers.

MATERIALS AND METHODS

Plant Material and Incubation Conditions—The barley (*Hordeum vulgare* L.) cultivar Himalaya (1985 harvests, obtained from Department of Agronomy and Soils, Washington University, Pullman, WA) was used throughout this study unless stated otherwise. The preparation and imbibition of the embryoless half-seeds were done as described (30).

Northern Analysis—Total RNA was isolated from aleurone layers using guanidine HCl as described (31). Ten μg of RNA was fractionated in a formaldehyde-agarose gel and blotted onto a Genescreen membrane (Du Pont-New England Nuclear). The blots were hybridized and washed according to the method of Church and Gilbert (32). Moist blots were sandwiched between plastic wrap and exposed to Kodak XAR-5 film with an intensifying screen at −70 °C.

Genomic Southern Analysis and Chromosomal Mapping—For the Southern analysis, genomic DNA was isolated from barley using a modified version of Sutton (33). Briefly, 25 g of liquid nitrogen frozen tissue was powdered in a mortar and then homogenized in 25 ml of extraction buffer (0.2 M Tris-HCl, pH 8.5, 10 mM EDTA, 1% SDS). The mixture was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged (12,000 × *g*). The aqueous was removed to a fresh tube, adjusted to 0.5 M $NaClO_4$, and extracted again with an equal volume of phenol:chloroform:isoamyl alcohol. After centrifugation at 12,000 × *g*, DNA was precipitated with two volumes of ethanol and spooled out with pipette. Spooled DNA was resuspended by overnight dialysis against TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)), treated with 50 μg/ml RNase A (37 °C, 1 h), adjusted to 150 mM NaCl, and extracted twice with chloroform:isoamyl alcohol (24:1). Genomic DNA was spooled from the final aqueous phase, dialyzed against TE, and stored at 4 °C. Hybridization was carried out as described under "Northern analysis."

Chromosomal mapping was conducted with genomic DNA isolated from euplasmic wheat-barley addition lines (34). Each line bears all wheat chromosomes plus a pair of barley chromosomes 1–4, 6, and 7. The additional line carrying barley chromosome 5 is not available due to sterility. Genomic DNA was also prepared from the progenitor barley (cv. Betzes) and wheat (cv. Chinese Spring) cultivars.

Construction and Screening of cDNA Libraries—λgt10 and λZapII libraries were constructed essentially as described (35) using poly(A)$^+$ RNA isolated from ABA treated aleurone layers. A 450-base pair cDNA (covering the 3′ end) was isolated from the λgt10 library by plus/minus screening. Probes used in screening were the first strand cDNA prepared with poly(A)$^+$ RNA isolated from barley aleurone layers treated with or without ABA. This partial cDNA clone was then used to screen the λZapII library, and a cDNA clone with the length of about 800 bp was obtained. Dideoxy chain termination sequencing reactions (36) were done using a Sequenase® version 2.0 sequencing kit (U. S. Biochemical Corp.). Editing and analysis of DNA and protein sequence were conducted with the DNA Inspector™ IIe program (37) and the GCG software package (38). Database comparisons (Genbank, EMBL, and PIR) were done using default search parameters of the FASTA, TFASTA, and BLAST programs from the GCG software package.

Isolation of HVA22 Genomic Clone—Genomic DNA was isolated as described above. Several partial genomic libraries were prepared. Originally, we attempted to clone a 6.2-kb *Hin*dIII fragment without success. Therefore, we took another strategy and cloned the gene as two neighboring *Bam*HI fragments, 3.2–3.5 kb in size. Total genomic DNA was digested to completion with *Bam*HI and electrophoresed in a 0.6% agarose gel with TAE buffer (40 mM Tris acetate, 2 mM EDTA). Fragments (2.9–3.6 kb) were excised, electroeluted, and partially filled in with Klenow fragment using dATP and dGTP. λZAP II (Stratagene) DNA was ligated with T4 DNA ligase, cut with *Xho*I, and partially filled in with dCTP and dTTP. A 1:1 molar ratio of arms (0.5 μg) to genomic inserts (0.05 μg) was ligated and packaged as instructed by the manufacturer. The library was screened with the 5′ and 3′ specific cDNA fragments. To sequence the clones, a series of nested deletions were generated with ExoIII nuclease. Sequencing was performed the same way as with the cDNA for both strands in the proximal promoter region (to approximately −250 bp), the transcribed region, and on one strand in other regions.

S1 Mapping, Ribonuclease Protection Asssay and Primer Extension—For S1 mapping, a *Pst*I/*Eag*I fragment (−846 to +95, Fig. 3) was isolated, dephosphorylated, and end-labeled. Hybridization, S1 digestion, and gel electrophoresis were done according to Khursheed and Rogers (39) except hybridization was done overnight. For ribonuclease protection assay (40), a *Bgl*I/*Eag*I fragment (−282 to +95) was transcribed with T3 RNA polymerase and hybridized to the total RNA from barley aleurone layers treated with ABA and cycloheximide. Primer extension was performed to verify the transcription start site. A 30-base oligonucleotide (5′-CCGTG ACTTG TAGTT GTAGG TGCCC TGTTG-3′, +111 to +140, Fig. 3) was synthesized at the DNA Chemistry Facility, Washington University. Oligonucleotide labeling with kinase, hybridization, and reverse transcription were done essentially as described (40). Total RNA (30 μg) isolated form aleurone layers treated with or without ABA and cycloheximide for 24 h was hybridized overnight at 30 °C with the ^{32}P-labeled oligonucleotide. *Escherichia coli* tRNA was also included as a negative control. Reverse transcription was conducted at 42 °C for two h. Extension products were electrophoresed and visualized by autoradiography as for DNA sequencing.

DNA Constructions and Transient Expression—The 3-kb HVA22 promoter plus the 46-bp untranslated sequence of HVA22 were linked to the coding sequence for the *E. coli GUS* gene (41) with a modified ATG initiation codon (42). The first intron, the second exon (only 27 bp), and the second intron of HVA22 were inserted between the promoter and *GUS* gene. Five bases of the exon sequences at the splicing sites were also included. 3′ region (*Sph*I/*Sph*I fragment) of HVA22 was attached to the 3′ end of the *GUS* gene in the plus orientation. Promoter deletion mutants, P*Dra*IIIGU, P*Pst*IIGU, P*Nar*IIGU, P*Bgl*IIGU, and P*Alu*I IGU were generated by recombining the appropriate restriction fragments. P*Dra*IIIG is different from P*Dra*IIIGU because the former has no 3′ region. There was no difference observed on the level of ABA induction among the constructs with or without the 3′ region and that with the 3′ region at an opposite orientation. There is an AATAAA signal downstream from the proximal cloning site on the vector, and we think that this sequence might function as a poly(A) addition signal for these constructs possessing no 3′ region. Therefore, to simplify the cloning and exclude any possible interactions between the intron and 3′ region for ABA induction, all constructs used in the analysis of intron had no 3′ region. These constructs were designated without a U at the end of their names to distinguish them from those with 3′ region. To make the construct PλG, two oligonucleotides were designed in such a way that they included a short stretch of the λ DNA sequence, 10 bases of the intron 1 splicing site sequences and restriction site sequences. The polymerase chain reaction (with λ DNA as template) product was used to replace the intron 1-exon 2-intron 2 fragment in the P*Dra*IIIG construct. The size of the two fragments was identical. To dissect the effect of the introns on the gene expression, intron 1, exon 2, or intron 2 was, respectively, inserted to another template construct, named PG, which only consisted of the promoter (P*Dra*II fragment) and GUS coding region. Introns 1 and 2 also included five nucleotides of the exon sequences at both 5′ and 3′ splicing sites to meet the sequence requirements for splicing. The conjunction sites of all constructs were confirmed by sequencing. DNA fragments derived from the polymerase chain reaction were sequenced completely to ensure that no unexpected mutations were introduced by the polymerase chain reaction process.

Particle bombardment, extract preparation of bombarded tissue, and luciferase and GUS assay were performed essentially as described by Lanahan *et al.* (42) except for the following modifications. Bombarded half-seeds were incubated with or without 10^{-5} M ABA for 24 h before being homogenized. Probably because of the need for the bombarded tissue to recover from wounding, it was determined that a 24-h incubation time was optimal for GUS expression. Glycerol (final concentration, 20%) was included in the grinding buffer as enzyme stabilizer. For GUS assay, samples were incubated at 37 °C for 20 h. Methanol (final concentration, 20%) was included in the buffer to minimize endogenous GUS activity present in many plant tissues (43). Sodium azide was added to a final concentration of 0.02% to the GUS assay mixture to suppress the growth of microorganisms. Extensive studies were conducted to optimize the GUS and luciferase assay conditions so that all assays were done within a linear range. A constitutively expressed construct, pAHC 18 (ubiquitin promoter-luciferase fusion) (44), served as the internal control throughout the study. To make sure that expression from the ubiquitin promoter was not affected by ABA, pAHC18 was delivered along with ^{35}S promoter/GUS chimeric gene construct pBI221 (41) to the half-seeds. GUS and luciferase activities were assayed for the extracts from seeds treated with or without ABA. Because ABA has little or no effect on the cauliflower mosaic virus ^{35}S promoter (45), we used the ^{35}S-driven GUS expression of pBI221 (41) to standardize the luciferase activity from pAHC18. Abscisic acid induction on the ubiquitin promoter of pAHC18 was hence calculated. The average from 16 duplicates was 1.04-fold. Moreover, Student's t test analysis indicated that the luciferase activity difference between the ABA-treated and the control samples was not significant ($p = 0.538$; $n = 16$).

RESULTS

Induction of HVA22 by ABA and Cycloheximide—As part of our effort to elucidate the action of ABA in barley aleurone layers, we have isolated more than 20 ABA-induced cDNA clones by plus/minus screening an aleurone layer cDNA library. Among these clones, HVA22 appears to be the least abundant one. Northern blot analysis indicated that HVA22 encodes an mRNA with the size of 800–900 bases (data not shown). It was induced by ABA in as short as 30 min (Fig. 1). The mRNA level appeared to reach the peak after 4–8 h of ABA treatment. Further ABA treatment led to a decrease of the mRNA abundance (Fig. 1*A*). It should be emphasized that the decrease of the mRNA at the later stages was not due to the degradation of ABA because the mRNA accumulation pattern remained the same when the buffer containing ABA was changed every 4 h (data not shown).

Cycloheximide, a protein synthesis inhibitor, also induced the expression of HVA22 to a significant level (Fig. 1*B, lanes 3-5*). However, the cycloheximide induction of HVA22 expression appeared to follow a kinetics slower than that of ABA induction; it took at least 4 h of incubation before any increase in the level of HVA22 mRNA was observed (Fig 1*B*). A synergistic effect was observed when the tissue was treated with both ABA and cycloheximide. The level of HVA22 mRNA in tissue treated with both inducers was much greater than the sum of the levels of treatment with either inducer (Fig. 1, *A* and *B*). At least one other protein synthesis inhibitor, emetin, had a similar effect on the induction of HVA22 mRNA (data not shown).

HVA22 Is a Single Copy Gene Located on Barley Chromosome 1—HVA22 appears to be a single copy gene based on genomic Southern analysis with reconstitution standards (Fig. 2*A*). A single *Hin*dIII band as well as a single *Xba*I band hybridized to the probe with an intensity similar to the single copy equivalent cDNA band (*lane 6*). There is a single *Bam*HI site in the middle of HVA22 cDNA, and genomic Southern analysis with *Bam*HI-digested DNA revealed two bands with about equal intensity (*lane 5*). This is also true for the *Eco*RV-digested DNA (*lane 3*), because there is a single *Eco*RV site in the transcribed region (in the intron 1, Fig. 3) of HVA22.

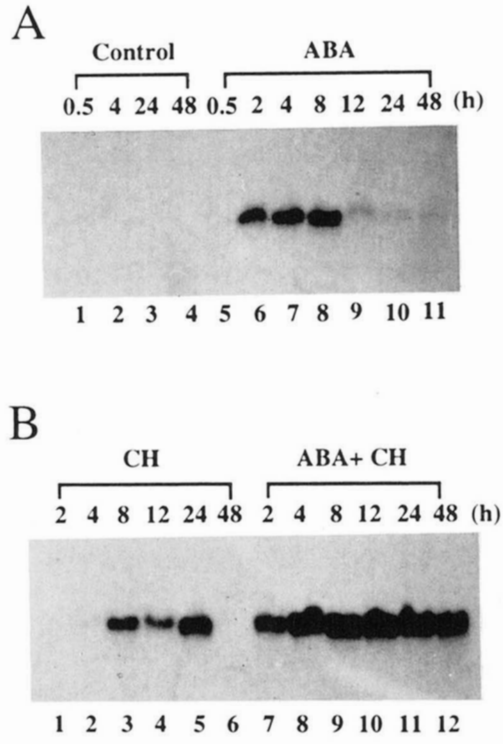


FIG. 1. **Northern blot analysis showing the time course of HVA22 gene expression regulated by ABA and cycloheximide.** *A*, treatments with ABA alone. RNA was prepared from aleurone layers incubated in seed buffer (see "Materials and Methods") with 10^{-5} M ABA (*lanes 5-11*) or in seed buffer alone (*lanes 1-4*) for the time (*h*) indicated. *B*, treatments with cycloheximide (*CH*) or combination of cycloheximide and ABA. Aleurone layers were incubated either with cycloheximide alone (10 μg/ml) *lanes 1-6*) or with cycloheximide plus ABA (*lanes 7-12*) for the time (*h*) indicated. The RNA blot was probed with the cDNA clone of HVA22.

Southern blot analysis was carried out using DNA isolated from wheat lines carrying additional barley chromosomes. As shown in Fig. 2*B*, the wheat line bearing barley chromosome 1 (*lane 3*) shares a common hybridization band with the barley progenitor (*lane 1*), indicating the localization of HVA22 on chromosome 1. Hybridization to the wheat homologs of HVA22 can be seen as common bands in the wheat progenitor and all of the addition lines.

Deduced Amino Acid Sequence of HVA22 Is Highly Homologous to That of Human DP1 Gene—The HVA22 cDNA sequence perfectly matched the corresponding genomic sequence. A typical poly(A) addition sequence AATAAA is present. There are two potential open reading frames in the 5′ to 3′ direction, with two ATG start codons separated by eight bases. Another open reading frame was found in the complementary strand. However, it is unlikely this open reading frame codes for any ABA-inducible protein, because the sense single strand DNA probe did not hybridize to any RNA isolated from the tissues treated with ABA (data not shown). Of the remaining open reading frames, the first one (designated *ORF C*, Fig. 4*A*) is more likely to be used *in vivo* based on the following experimental results. *In vitro* transcription and translation of the cDNA generated a 15-kDa peptide. When the first ATG (Fig. 3, position +178; Fig. 4*A*, *lanes 3* and *4*; Fig. 4*A*) or both of the ATGs (*lane 9*) were replaced by the oligonucleotide-directed mutagenesis, no such peptide was produced. However, mutation of the second ATG (Fig. 3, position +189) (*ORF B*) did not abolish the production of the 15-kDa peptide (*lanes 5* and *6*). Therefore, we believe that the first open reading frame is the one used *in vivo*.

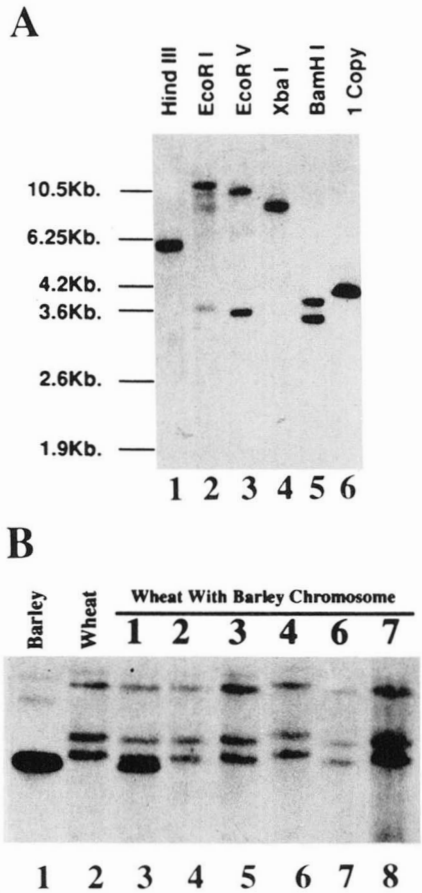


FIG. 2. **Genomic Southern analysis showing HVA22 is a single copy gene located on chromosome 1.** Southern blot analysis (*A*) and chromosome mapping (*B*) of HVA22 were conducted as described under "Materials and Methods." Ten μg of genomic DNA was digested with the restriction enzymes as indicated. One copy equivalent of HVA22 cDNA (*lane 6*) was loaded for intensity comparison. For chromosome mapping, 10 μg of DNA isolated from wheat lines carrying additional barley chromosomes 1, 2, 3, 4, 6, and 7 was digested with *Hin*dIII separately. The Southern blot was probed with HVA22 cDNA insert. Because wheat is hexaploid, it is expected to have three HVA22 homologs.

The HVA22 protein (see Fig. 3) has a charged carboxyl-terminal region where 20 out of 37 amino acids are potentially charged, and there may be a net positive charge of eight amino acids. Adjacent to this positively charged region is a sequence (KGAS) that could be phosphorylated by a serine kinase (46). The middle region of the protein (amino acids 45–85) is relatively hydrophobic and leucine rich. At the COOH terminus, there are four properly spaced histidine residues that could form a potential coordination complex with a metal ion as found in many "zinc finger" proteins.

GenBank search indicates that HVA22 is highly homologous to human DP1 gene, with a 29% identity and a 54% chemical similarity at the amino acid level (Fig. 4*B*). The significance of homology was further analyzed with another alignment program (47). In this type of analysis, a large number (usually 100) of sequences are randomly generated with the same amino acid composition of the two sequences to be compared. Then, the maximum score for this pair of real sequences is compared with the distribution of the maximum scores for those randomly generated sequences. The alignment score is the number of standard deviations by which the maximum score for the real sequences exceeds the average maximum score for those randomly generated sequences (47). This analysis reveals the homology between *HVA22* and Human *DP1* gene is significant, with a high alignment score of 9, indicating the probability of random sequences to have such a high score is less than one in 10^{19} (47).

G Box-like Sequences Are Found in the Promoter Region of HVA22—We have sequenced more than 1.5 kb in the 5′ untranscribed region and about 1 kb in the 3′ untranscribed region (Fig. 3). Four introns divide the transcribed portion of the gene. All are internally bordered by the dinucleotide pairs, 5′-GT. . .AG-3′ (48, 49). Exon 2 is extremely small, only 27 bases long. A 14-bp perfect inverted repeat was found in the 5′ untranscribed region (−653 to −666 *versus* −894 to −907). This inverted repeat could be extended to be a 20-bp imperfect (18/20) repeat. The distal part of the inverted repeat (−894 to −907) has a 13-bp direct repeat about 80 bases upstream (−981 to −993). Moreover, an 8-bp element, CACGTGTC, is present within 250 bp upstream of the mapped transcription start site. This element generally matches the proposed ABRE (CACGTGGC) (29). However, the second residue (counting from the right) of the element in HVA 22 is a T, in contrast to a G residue in the G box of the light-inducible gene and ABRE of wheat (*Em, Triticin*) (29). Finally, a G box-like element is located in the −80 region but in an opposite orientation (cCGCCACGTAC). Multicopies of its imperfect (10/11) inverted counterpart (GTACGTGGCGc) have been shown to be able to direct ABA response in a chimeric promoter (45).

Mapped Transcription Start Site Is Downstream of HVA22 cDNA Start Site—The primer extension data suggest that the transcription of HVA22 starts 87 bases downstream of the first nucleotide of the cDNA (Fig. 5*A*, *lane 5*). S1 ribonuclease assay (Fig. 5*B*, *lane 7*) confirmed the result of the primer extension. Puzzlingly, this 87-bp fragment perfectly matches with the corresponding genomic sequence, indicating that it is not due to a cloning artifact. To further confirm the result, we conducted a ribonuclease protection assay (Fig. 5*B*, *lanes 3-5*). The *Bgl*I/*Eag*I fragment (−282 to +95, Fig. 3), related to the mapped transcription starting site, was transcribed with T3 RNA polymerase. This antisense probe was hybridized to the total RNA from barley aleurone layers treated with ABA and cycloheximide. A fragment with the size of 100 bases (compared with the DNA sequencing size mark) was protected from the digestion of ribonuclease A and T1 (Fig. 5*B*). Because the RNA migrates approximately 5–10% slower than DNA of the same size (50), the data is consistent with the S1 mapping result. Several bigger bands were observed when 10 times more probe was used (Fig. 5*B*, *lanes 4* and *5*). However, they appear to be the partially degraded RNA probe, because similar bands were also present in the *E. coli* tRNA sample (data not shown).

ABA Response Complex Consists of at Least Three Elements—Because barley cannot be stably transformed, we performed transient expression assays for the promoter analyses. The test constructs were delivered into barley half-seeds with particle bombardment as described under "Methods and Materials." Because of the inherent variability of transfection efficiencies, an oat ubiquitin promoter/firefly luciferase reporter construct (44) was cotransfected as an internal control. The luciferase promoter was shown not to be significantly affected by ABA (see "Materials and Methods"). Therefore, the measured GUS activity of one construct could be normalized with luciferase activity from the same shot.

The comparison of dosage response of HVA22 mRNA with that of GUS expression from the construct, P*Dra*IIIGU, indicated that their patterns were extremely similar (Fig. 6*A*). The gene responded to ABA at a level as low as 10^{-9} M. An increase of ABA concentration resulted in an almost linear

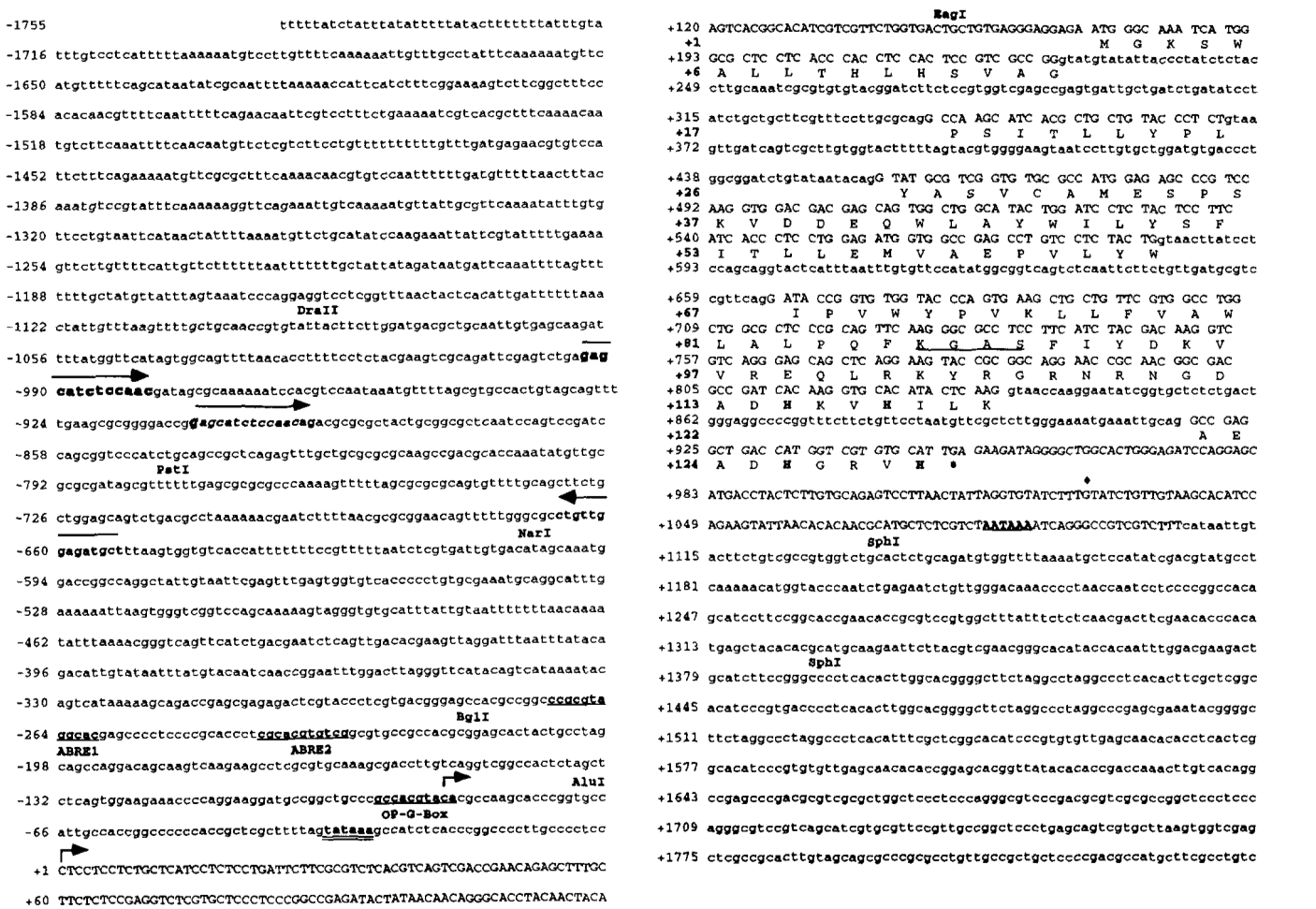
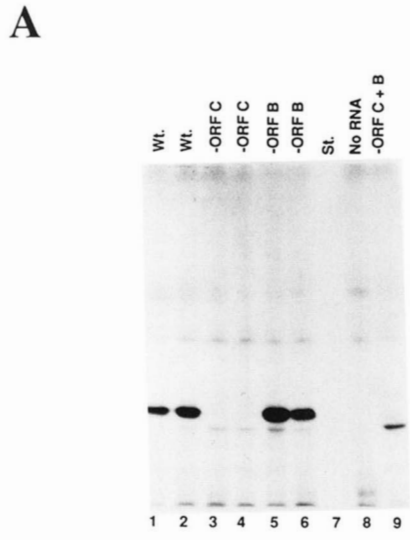


FIG. 3. **Nucleotide sequence and deduced amino acid sequence of *HVA22* gene.** Promoter, introns, and 3′ flanking region are indicated with *lower case letters*. Exons are shown in *upper case letters*. A 13-base direct repeat (in *outlined letters*) and a 14-base inverted repeat (in *boldface letters*) in the promoter region are marked with *arrows*. The putative TATA box is in *boldface double underlined letters*. HVA22 might have two transcription start sites (see "Results"). Nucleotide +1 is the mapped transcription start site, and nucleotide −86 corresponds to the 5′ end of the cDNA sequence. The location of the poly(A) tail addition in the 3′ untranslated regions is shown by a *solid diamond* (♦) and consensus polyadenylation sequence (AATAAA) is in *boldface* and *underlined*. The position of restriction sites, which were used to make constructs, is labeled. The amino acid sequence, which could be phosphorylated by a serine kinase, is *underlined*. The four histidines in *boldface* have the potential to form a zinc finger structure.

increase of both HVA22 mRNA levels and GUS activities until the concentration reached 10^{-6} M. Higher ABA concentrations led to a nonlinear enhancement of the expression. The similarity of the expression pattern at the mRNA (HVA22) and the protein (GUS) level suggests that the expression of HVA22 is most likely regulated at the level of transcription.

To define the ABA response elements of HVA22, we first analyzed the 5′ deletion constructs. As shown in Fig. 6*B*, the absolute GUS activities and the level of ABA induction were not significantly reduced until the 5′ sequence was deleted to about 0.3 kb (P*Bgl*IIGU). It appears that between −671 (*Nar*I site) and −282 (*Bgl*I site) lies an element important for an ABA response. The ABA response was almost abolished when the promoter was deleted to −136-bp region (*Alu*I site). Detailed analysis indicates that two elements located between *Bgl*I and *Alu*I sites share certain homology with ABRE defined previously (27, 29). The first element, CCGCGTAGGCAC (−271 to −260), is less similar than the second element, GCACGTGTCGG (−240 to −229), to the ABRE of *Em* (GGACACGTGGC) (29) and *Rab 16* (GTACGTGGCGC)(27) genes. ABRE of the *Em* gene, was necessary for ABA response and was found to interact with a leucine zipper protein (29). In our study, deletion of the region containing the first element (designated ABRE 1) alone resulted in a significant reduction (from 11X to 6X). Further deletion to −229 (the end of the ABRE 2) led to an ABA induction of only 4X, which is very close to 3X from *Alu*I (−130) promoter fragment (Fig. 6*C*). Therefore, it seems that there are at least two elements in the 5′ untranscribed region that are important for the ABA response of HVA22.

However, the ABA response of HVA22 requires other element(s) in addition to those located in the 5′ untranscribed region. Initially, the high level of ABA induction of P*Dra*IIIG was only observed when the intron 1-exon 2-intron 2 fragment of HVA22 was included in the construct. There have been reports that introns have general enhancer activities (51), yet we found that introns/exon fragment affected not only the absolute level of GUS expression but also the level of ABA induction. If no insert was placed between the GUS coding region and the 5′ promoter region (the construct PG), ABA induction was reduced to 5X (Fig. 6*D*). This result does not necessarily suggest that the insert accounted for the high level of gene expression because the spacing between the 5′ pro-



B

```
HVA 22    1 MGKSWALLTHLHSVAGPSITLLYPLYASVC
              :.  : ::..  |: .  |.: || | |:
 DP 1    40 LVALYLVFGYGASLLCNLIGFGYPAYISIK
                    .           .         .
HVA 22   31 AMESPSKVDDEQWLAYWILYSFITLLEMVA
            |:|||.| ||.|||.||::|:.:.: |:..
 DP 1    71 AIESPNKEDDTQWLTYWVVYGVFSIAEFFS
                    .           .         .
HVA 22   61 EPVLYWIPVWYPVKLLFVAW.LALPQFKGA
            : .| |:|.:| :|   |: | :| .. .||
 DP 1   101 DIFLSWFPFYYMLKCGFLLWCMAPSPSNGA
                    .           .         .
HVA 22   90 SFIYDKVVRE QLRKYRG......RNRNGDA
            .::|.:::|. :  |. :       :: .:.|
 DP 1   131 ELLYKRIIRP FFLKHESQMDSVVKDLKDKA
                    .
HVA 22 114 DHKVHILKAEADHGRVH
           ..... :. ||..: |:
 DP 1   161 KETADAITKEAKKATVN
```

FIG. 4. *A*, determination of the HVA22 open reading frame with an oligo-directed mutagenesis technique and wheat germ extract. Oligo-directed mutagenesis was performed as described (74). The products of *in vitro* transcription followed by *in vitro* translation of "wild type" (*Wt*) (*lanes 1* and *2*), ORF C mutant (*lanes 3* and *4*), ORF B mutant (*lanes 5* and *6*), no RNA (*lane 8*), or ORF B and ORF C mutants (*lane 9*) were analyzed by SDS-polyacrylamide gel electrophoresis (75). *B*, comparison of the deduced amino acid sequence of barley HVA22 and human DP1 genes. *Vertical lines* denote identities, *double dots* are conservative replacements, and *single dots* indicate a low degree of similarity above random mean value (38).

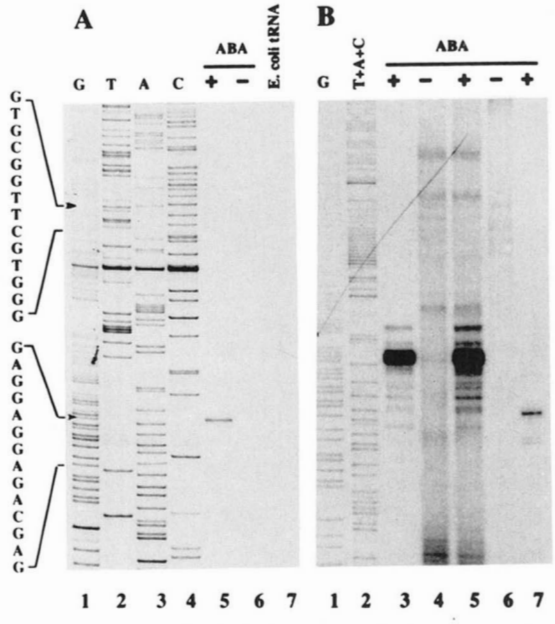


FIG. 5. **Mapping of HVA22 transcription start sites.** *A*, primer extension analysis of HVA22 mRNA. Total RNA isolated from aleurone layers treated with 10^{-5} M ABA (*lane 5*) or without hormone (*lane 6*) was mapped by primer extension. *E. coli* tRNA (*lane 7*) was included as a negative control. The sequencing ladder (*lanes 1-4*) came from a sequencing reaction with the same primer as that used in the primer extension. The first G of the top stretch sequence corresponds to the first C of HVA22 cDNA. The first G of the bottom stretch represents the mapped transcription start site. *B*, ribonuclease protection assay (*lanes 3-5*) and S1 nuclease mapping (*lanes 6* and *7*). Thirty μg of total RNA isolated from the aleurone layers treated with ABA (*lanes 3* and *5*) or from the control (buffer only) (*lane 4*) was hybridized with the ^{32}P-labeled RNA probe (4×10^8 dpm/pmol) transcribed from the *BglI*/*EagI* genomic fragment of HVA22 (see Fig. 3), digested with ribonucleases as described under "Materials and Methods." Probe used in the samples for *lanes 4* and *5* was 10 times higher than that for *lane 3*. For S1 nuclease mapping, total RNA was annealed to the kinase-labeled HVA22 *PstI*/*EagI* genomic fragment (see Fig. 3). S1-resistant digestion product is shown in *lane 7*. The size markers (*lanes 1* and *2*) were from an irrelevant sequencing reaction.

moter and the GUS coding sequence might be critical for the high level of gene expression. To address this question, we prepared a λ phage DNA fragment, which was flanked by both the 5′ and 3′ splicing junction sequences of HVA22 intron 1 and was the same length as the introns/exon fragment present in P*Dra*IIIGU. Introduction of this fragment to the construct PG (PλG) failed to enhance its ABA response. Therefore, we believe that there is information located in introns/exon fragments of the constructs necessary for the ABA response of HVA22. To define the region(s), we cloned the intron 1, exon 2, and intron 2 fragment into the construct PG separately. The analysis of these constructs suggests that intron 1 alone has a dramatic effect on the ABA induction of HVA22. In contrast, the effect of intron 2 was much smaller, and that of exon 2 was negligible. Because we have demonstrated that the spacing between the 5′ promoter region and GUS coding region is not crucial in the regulation of the GUS gene expression of our test constructs, the differences mentioned above were unlikely the consequence of the length differences among these inserts.

DISCUSSION

Abscisic acid appears to play an important role in plant development, especially at the transition of seed development and germination, and in the plant's response to environmental stresses, yet very little is known concerning the mode of action of this hormone. Circumstantial evidence has led to the suggestion that ABA exerts its regulatory role by altering the pattern of gene expression and/or the function of membranes. Several types of ABA-induced genes have been isolated and characterized in recent years, yet the function of most of them is still not clear (3). The barley *HVA22* gene that we have studied has several unique features: 1) sequence homology to a human gene related to colon cancer development and structural features resembling known regulatory proteins; 2) superinduction by cycloheximide; 3) two potential transcription start sites; 4) a hormone response element located in the first intron. Barley HVA22 appears to be a single copy gene on chromosome 1, thus some of the unique features, such as the two potential transcription start sites, cannot be attributed to the presence of multiple copies of homologous genes.

Although the function of HVA22 is not known, the polypeptide encoded by this gene contains features shared by some

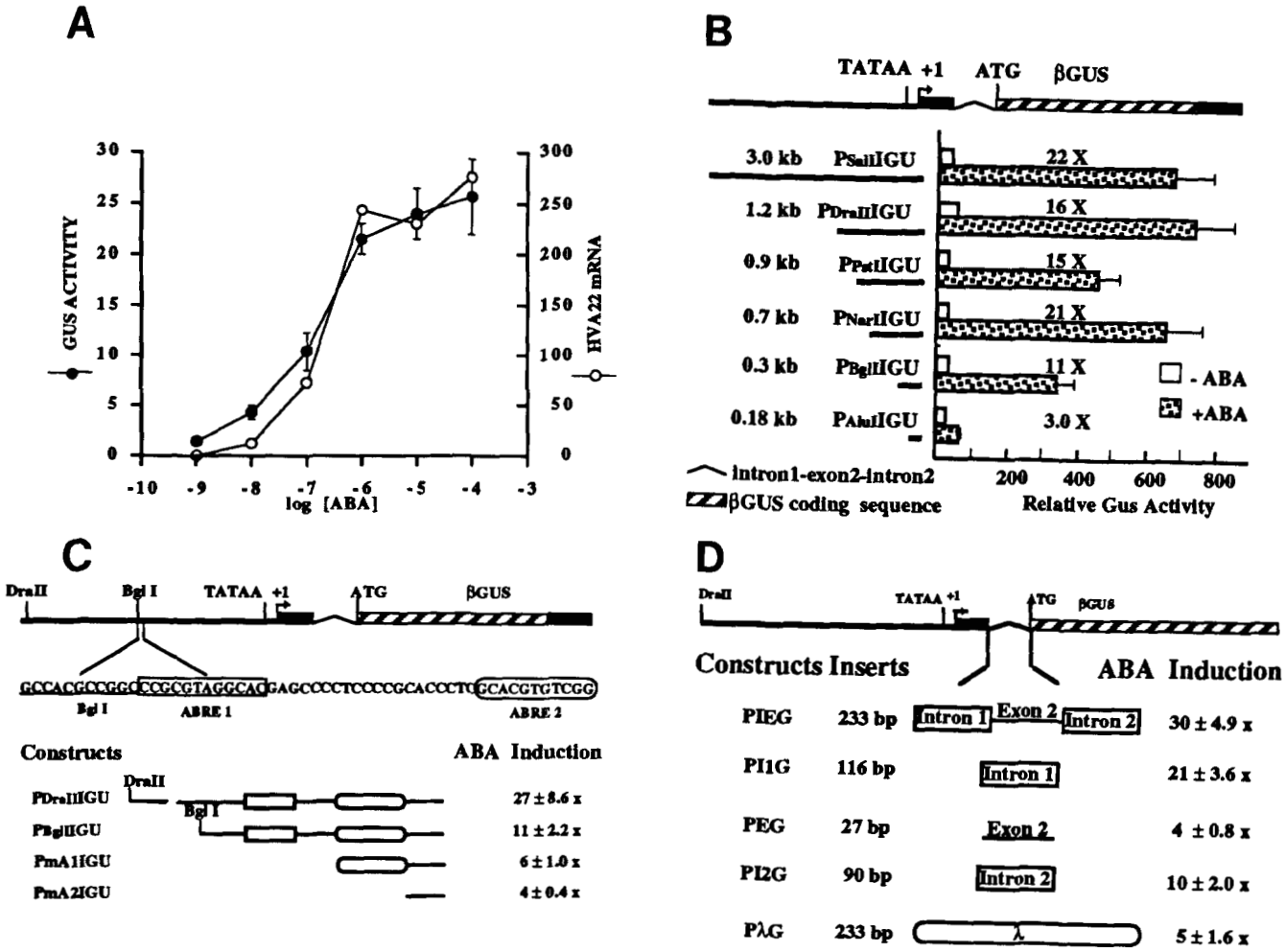


FIG. 6. **Functional analysis of ABA response complex of HVA22 promoter and intron fragments.** *A*, dosage response curve of ABA-inducible HVA22 RNA accumulation (○) and dosage response of *GUS* gene expression driven by HVA22 promoter (●). For the Northern analysis, RNA was isolated from mature imbibed aleurone layers treated with ABA at the concentrations of 10^{-9} to 10^{-4} M, respectively. Correspondingly, protein extract was prepared from mature half-seeds treated with or without 10^{-9} to 10^{-4} M ABA for 24 h, after the seeds having been shot with P*Dra*IIIGU and the internal control pAHC18 constructs. The ABA induction was expressed as the ratio of normalized GUS activity of the samples treated with ABA over that of those incubated with buffer only. Each point represents the mean of at least six replicas. Northern blot analysis of HVA22 mRNA was performed as described under "Materials and Methods." Each lane was loaded with five μg of total RNA prepared from the aleurone layers treated without (control) or with 10^{-9} to 10^{-4} M ABA. The autoradiography was quantified with a computing densitometer (model 300A, Molecular Dynamics, California). *B*, 5′ deletion analysis of HVA22 promoter. A schematic diagram of testing construct is shown at the *top*; the *thin black angled line* indicates the position of the first intron-second exon-second intron fragment of HVA22 inserted between the the 5′ untranslated sequence (*thick black line*) and the GUS coding sequence (*hatched box*). The 3′ untranslated/untranscribed region (*thick black line* to the *right* of the *hatched box*) was from the *SphI/SphI* genomic fragment including polyadenylation sequence (AATAAA). Promoter deletion was conducted by recombined restriction fragments (see Fig. 3 for the location of the restriction sites). To the *right* are results obtained from expressing each construct. The *white box* stands for the relative GUS activity of the samples from layers incubated in the absence of ABA, and the *stippled box* represents those from the layers incubated in the presence of ABA. The relative GUS activity of each construct is the mean of at least eight replicas. *Error bar* indicates the standard error of each set of replicas. *X* indicates the fold of the increase. *C*, defining of ABA response cis-acting elements in the 5′ promoter region. Sequence shown is the region in which oligo-directed mutagenesis was conducted. ABRE1 and ABRE2 strand for ABA response element 1 and 2, respectively. ABA induction fold (with SE) represents the mean of at least eight replicas. *D*, the effect of the introns on HVA22 induction by ABA. Introns in the constructs included their own intron slicing sequences (five bases of exon plus five bases of intron at the intron/exon junction). The insert between the 5′ untranslated region and the GUS coding region of PλG consists of a piece of λ fragment plus the 5′ and 3′ splicing sequence of intron 1 of HVA22. ABA induction fold (means of at least five replicas) of each testing construct was shown on the *right* column with standard errors.

DNA-binding proteins. The four spaced histidine residues near the COOH terminus are properly spaced to form a potential coordination complex with divalent ions such as Zn^{2+}. However, all of the reported "zinc finger" structures have at least one cysteine, thus whether the HVA22 protein indeed has a zinc finger structure remains to be tested. The COOH-terminal region is also strongly positively charged, suggesting the possibility that HVA22 could possibly interact with negative-charged nucleic acids. An ABA-induced RNA-binding protein in maize has been reported (52) yet there is no homology between HVA22 and this maize protein. The HVA22 protein also has a sequence that could possibly be phosphorylated (KGAS) by a serine kinase (46). In addition, there is an unusual N-G peptide linkage that has been shown to be labile at pH levels higher than 7.4 (53, 54). Preliminary evidence indicates that the HVA22 protein appears to turn-over quite fast in barley aleurone cells;[2] the instability may be attributed to the presence of this N-G linkage. Although

[2] S. J. Uknes, Q. Shen, and T.-H. D. Ho, unpublished observations.

HVA22 homologs have been found in all of the cereals so far studied,[3] the high degree of sequence homology between HVA22 and human DP1 is surprising because of the apparent evolution divergence between these two organisms. Human DP1 acts early in the process of colon carcinogenesis (55) Northern analysis, and sequencing of cDNA isolated from a fetal brain cDNA library indicated that this gene codes for a large 3.5 kb-mRNA, in contrast to the 0.9-kb HVA22 mRNA. However, reading frame analysis suggests that the translation product of the DP1 gene is only 185 amino acids, similar to that of HVA22 protein (130 amino acids). Interestingly, the DP1 protein also contains one unusual N-G linkage as in HVA22. Both ABA and retinoic acid, an important animal hormone, are derived from carotenoids and share structure similarities. Furthermore, ABA has been found in mammalian brain tissues (56). Although it is tempting to suggest a similar regulatory role for both barley HVA22 and human DP1, it should be pointed out that it is also possible that, despite the apparent sequence homology between these two proteins, they have completely different types of function. Nonetheless, given the similarities between these two proteins and the fact that ABA and ABA-like compounds are present in mammalian tissues, it would be extremely interesting to determine the function of these proteins.

The expression of HVA22 in the aleurone tissue is induced by either ABA or protein synthesis inhibitors, and a synergistic effect is observed when both types of inducers are present. The timing of ABA induction has a short lag (less than 30 min), and it appears to be transient. This type of expression pattern is common among many regulatory molecules such as transcription factors. Cycloheximide superinduction of several transcription factors has also been reported (57–60), although the mechanism of cycloheximide superinduction has not been explored. It is plausible that cycloheximide prevents the movement of ribosomes relative to mRNA, hence loading up the mRNA with ribosomes, rendering it less sensitive to a constitutive ribonuclease. It is also possible that cycloheximide treatment leads to stabilization of HVA22 mRNA by inhibiting the synthesis of a ribonuclease. Furthermore, in the case of c-*fos* and c-*jun*, it has been observed that the effect of cycloheximide at subinhibitory concentrations is actually at the level of transcription (61). In plants, a set of genes (*SAUR*) regulated by the phytohormone, auxin, can also be induced by cycloheximide at a post-transcriptional level (62).

One of the most intriguing features of HVA22 is that its transcription start site, as unequivocally mapped by three different techniques, appears to be downstream from the 5′ end of the longest cDNA clones. Because the cDNA sequence matches perfectly with that in the genomic clone except regions where the introns are located, it is unlikely that the cDNA sequence is artifactual due to some sort of DNA rearrangement during cloning. Several HVA22 cDNA clones were isolated in the course of this work, yet only one appeared to contain sequences beyond the mapped transcription start site. We would like to suggest that there are two transcription sites for HVA22, one is routinely used and the other one, an upstream one, only very rarely used. Other examples of this have been reported. For example, a carrot gene encoding a hydroxyproline-rich glycoprotein has been shown to have two transcription start sites (63). Wounding stimulates transcription from both sites (63). While ethylene treatment suppresses transcription from the downstream site it increases transcription from the upstream one (64). Attempts have been made without success to determine whether the upstream transcription start site is used in particular cell types under specific induction conditions, for example, cycloheximide superinduction. It is possible that the putative upstream site is so infrequently used that the presence of the transcript cannot be detected using conventional techniques. The downstream transcription site is proceeded with a standard TATA box at −34, yet no similar box is present near the putative upstream transcription site. Several TATA-less promoters for class II genes have been reported in recent years (65, 66), but there does not appear to be any common features shared among these genes. The significance of having the putative upstream transcription start site in *HVA22* gene needs to be further explored in future projects.

The level of GUS expression in our transient expression studies as a response to treatment with various ABA concentrations is tightly correlated with the levels of HVA22 mRNA. Our observations strongly suggest that the ABA induction of HVA22 is likely to be at the level of transcription; however, mRNA processing and/or stabilization as a possible mechanism cannot be totally ruled out. The regulation of at least two other genes by ABA, the wheat *Em* gene (28) and rice *Rab 16* (27), has been shown to be at the transcriptional level. Because the presence of the HVA22 3′ untranslated region is not essential for the expression of GUS, additional analyses have been centered around the promoter region and the introns. In the promoter region, all of the essential elements appear to reside between −671 (*NarI* site) and lms136 (*AluI* site). Deleting from −671 to −282 (*BglI* site) reduced the ABA induction level by about 50%, yet further deletion to −136 virtually eliminates all of the responses to ABA. Two putative ABREs, similar to what has been reported (27, 29), are present between −282 and −136. Deletion of the upstream ABRE significantly reduces the effect of ABA, yet removal of both of these elements does not further reduce the residual ABA response. It is intriguing to note that the upstream ABRE appears to be less similar to the sequence defined by Mundy *et al.* (27) and by Guiltinan *et al.* (29) than the proximal one. Furthermore, a G box-like element (67) is located at −90 region in an opposite orientation (OP-G box). It is not clear whether both ABREs and the OP-G box need to be present to confer ABA response.

Replacing intron 1-exon 2-intron 2 with an unrelated sequence from λ phage appears to abolish all of the ABA response. Because the λ sequence is exactly the same length and also flanked by intron splicing junctions derived from intron 1 of HVA22, our observations clearly indicate that it is the sequence rather than the length of intron construct that is essential for the ABA response. Further analysis has revealed that the intron 1 sequence alone is sufficient to restore most of the ABA response. Because we have not shown that the effect of intron 1 is at the level of transcription, our observations have to be interpreted with caution. We have not ruled out the possibility that the presence of intron 1 facilitates the post-transcriptional processing of mRNA precursors. Therefore, the exact role of intron 1 in barley HVA22 gene awaits further investigations. It has been shown on many occasions that introns contain general enhancer functions (for example, see Ref. 52). Although deleting introns from our construct causes a reduction in the absolute level of GUS expression, the response to ABA treatment in terms of fold of induction is even more affected. Thus, we suggest that in addition to the general enhancer function, the first intron of HVA22 contains elements that are essential for the maximal response to ABA treatment. Recently, hormone response elements have been shown to be present in the introns of several mammalian genes (68–70). In the case of the chicken estrogen-responsive very low density apolipoprotein II gene,

[3] Q. Shen and T.-H. D. Ho, unpublished observations.

in addition to four elements located in the promoter region, two distinct regions in the first intron also appear to be involved in the hormone-regulated gene expression (71).

In summary, it appears that at least three elements, two in the promoter region and one in the first intron, are essential for the maximal ABA induction of barley *HVA22* gene expression. It is not surprising that more than one element is involved, because the expression of HVA22 appears to be under hormonal, developmental, and tissue-specific regulation as we have presented in this work. It is plausible that the maximal expression of HVA22 is the consequence of all of these elements interacting with specific protein factors in a coordinated manner. Complex promoter elements have been reported in both animal and plant genes. The complex glucocorticoid response element in animal appears to interact with both glucocorticoid receptor protein and AP-1 (72). The induction of barley α-amylase gene by another phytohormone, gibberellic acid, also appears to be the consequence of multiple factors interacting with a complex of promoter elements (42). Besides the gibberellin response element, three other elements are also involved in the regulation of α-amylase expression, and one of them appears to be part of the so called "endosperm box," a sequence known to regulate tissue-specific gene expression in plant seeds (73). It is likely that a complex set of elements are also responsible for the proper expression of barley HVA22. As part of our effort to investigate the molecular mechanisms underlying the interactions among these elements, linker scan mutagenesis experiments with HVA22 are currently underway.

Acknowledgments—We thank Gregory Heck and Bimei Hong for providing precious RNA samples used in this study and for advice and technical help. We also thank Dr. John Rogers and Sally Rogers for consultations in DNA sequence analysis and technical help. Other people in Dr.Ho's lab also contributed to the improvement of this paper in various ways.

REFERENCES

1. Singh, N. K., LaRosa, P. C., Handa, A. K., Hasegawa, P. M., and Bressan, R. A. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 739–743
2. Zeevaart, J. A. D., and Creelmann, R. A. (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39,** 439–473
3. Skriver, K., and Mundy, J. (1990) *Plant Cell* **2,** 503–512
4. Naumann, R., and Dorffling, K. (1982) *Plant Sci. Lett.* **27,** 111–117
5. King, R. W. (1976) *Planta* **132,** 43–51
6. Karssen, C. M., Brinkhorst-van der Swan, D. L. C., Breekland, A. E., and Korneef, M. (1983) *Planta* **157,** 158–165
7. Dure, L., III, Crouch, M., Harada, J., Ho, T.-H. D., Mundy, J., Quatrano, R. S., Thomas, T., and Sung, Z. R. (1989) *Plant Mol. Biol.* **12,** 475–486
8. Hong, B., Uknes, S. J., and Ho, T.-H. D. (1988) *Plant Mol. Biol.* **11,** 495–506
9. Bartels, R. J., Singh, M., and Salamini, F. (1988) *Planta* **175,** 485–492
10. Varner, J. E., and Ho, T.-H. D. (1976) in *The Molecular Biology of Hormone Action* (Papaconstantinou, J., ed) pp. 713–794, Academic Press, New York
11. Nolan, R. C., Lin, L.-S., and Ho, D. T.-H. (1987) *Plant Mol. Biol.* **8,** 13–22
12. Chen, H. H., Li, P. H., and Brenner, M. L. (1983) *Plant Physiol.* **71,** 362–365
13. Lachno, E. R., and Baker, D. A. (1986) *Physiol. Plant.* **68,** 215–221
14. Wright, S. T. C., and Hiron, R. W. P. (1969) *Nature* **224,** 719–720
15. Mundy, J., and Chua, N.-H. (1988) *EMBO J.* **7,** 2279–2286
16. Vilardell, J., Goday, A., Freire, M. A., Torrent, M., Martinez, M. C., Torne, J. M., and Pages, M. (1990) *Plant Mol. Biol.* **14,** 423–432
17. Bandziulis, R. J., Swanson, M. S., and Dreyfuss, G. (1989) *Genes & Dev.* **3,** 431–437
18. Bartels, D., Engelhardt, K., Roncarati, R., Schneider, K., Rotter, M., and Salamini, F. (1991) *EMBO J.* **10,** 1037–1043
19. Bornman, C. H., and Jansson, E. (1980) *Physiol. Plant.* **48,** 491–493
20. Johnson-Flanagan, A. M., Zhong, H., Thiagarajah, M. R., and Saini, H. S. (1991) *Plant Physiol.* **95,** 1044–1048
21. Chen, T. H. H., and Gusta, L. V. (1983) *Plant Physiol.* **73,** 71–75
22. Lin, L. S., and Ho, T.-H. D. (1986) *Plant Physiol.* **82,** 289–297
23. Jacobsen, J. V., and Shaw, D. C. (1989) *Plant Physiol.* **91,** 1520–1526
24. Leah, R., and Mundy, J. (1989) *Plant Mol. Biol.* **12,** 673–682
25. Mundy, J., and Rogers, J. C. (1986) *Planta* **169,** 51–63
26. Hornberg, C., and Weiler, E. W. (1984) *Nature* **310,** 321–324
27. Mundy, J., Yamaguchi-Shinozaki, K., and Chua, N.-H. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 1406–1410
28. Marcotte, W. R., Jr., Russell, S. H., and Quatrano, R. S. (1989) *Plant Cell* **1,** 969–978
29. Guiltinan, M. J., Marcotte, W. R., Jr., and Quatrano, R. S. (1990) *Science* **250,** 267–271
30. Belanger, F. C., Brodl, M. R., and Ho, T.-H. D. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1354–1358
31. Koehler, S. M., and Ho, T.-H. D. (1990) *Plant Cell* **2,** 769–783
32. Church, G. M., and Gilbert, W. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 1991–1995
33. Sutton, W. D. (1974) *Biochim. Biophys. Acta* **366,** 1–10
34. Islam, A. K. M. R., Shepherd, K. W., and Sparrow, D. H. B. (1981) *Heredity* **46,** 161–174
35. Watson, C. J., and Jackson, J. F. (1985) in *DNA Cloning Techniques: A Practical Approach* (Glover, D. M., ed) pp. 79–88, IRL Press, Washington D. C.
36. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
37. Gross, R. H. (1986) *Nucleic Acids Res.* **14,** 591–596
38. Devereux, J., Haeberli, P., and Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395
39. Khursheed, B., and Rogers, J. C. (1988) *J. Biol. Chem.* **263,** 18953–18960
40. Gilman, M. (1989) in *Current Protocols in Molecular Biology* (Ausubel, F. M., ed) pp. 4.7.1–4.7.8, John Wiley and Sons, New York
41. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987) *EMBO J.* **6,** 3901–3907
42. Lanahan, M. B., Ho, T.-H. D., Rogers, S. W., and Rogers, J. C. (1992) *Plant Cell* **4,** 203–211
43. Kosugi, S., Ohashi, Y., Nakajima, K., and Arai, Y. (1990) *Plant Sci.* **70,** 133–140
44. Bruce, W. B., Christensen, A. H., Klein, T., Fromm, M., and Quail, P. H. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9692–9696
45. Skriver, K., Olsen, F. L., Rogers, J. C., and Mundy, J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7266–7270
46. Kemp, B. E., and Pearson, R. B. (1990) *Trends Biochem. Sci.* **15,** 342–346
47. Dayhoff, M. O., Barker, W. C., and Hunt, L. T. (1983) *Methods Enzymol.* **91,** 524–545
48. Brown, J. (1986) *Nucleic Acids Res.* **14,** 9549–9559
49. Mount, S. M. (1982) *Nucleic Acids Res.* **10,** 459–472
50. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* pp. 7.71–7.78, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
51. Callis, J., Fromm, M., and Walbot, V. (1987) *Genes & Dev.* **1,** 1183–1200
52. Ludevid, M. D., Freire, M. A., Gomez, J., Burd, C. G., Albericio, F., Giralt, E., Dreyfuss, G., and Pages, M. (1992) *Plant J.* **2,** 999–1003
53. Stephenson, R. C., and Clark, S. (1989) *J. Biol. Chem.* **264,** 6164–6170
54. Bornstein, P. (1970) *Biochemistry* **9,** 2408–2421
55. Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., Sargeant, L., Krapcho, K., Wolff, E., Burt, R., Hughes, J. P., Warrington, J., McPherson, J., Wasmuth, J., Le Paslier, D., Abderrahim, H., Cohen, D., Leppert, M., and White, R. (1991) *Cell* **66,** 601–613
56. Le Page-Degivry, M.-T., Bidard, J.-N., Rouvier, E., Bulard, C., and Lazdunski, M. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 1155–1158
57. Milbrandt, J. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 4789–4793
58. Greenberg, M. E., Hermanowski, A. L., and Ziff, E. B. (1986) *Mol. Cell. Biol.* **6,** 1050–1057
59. Shaw, P. E., Schroter, H., and Nordheim, A. (1989) *Cell* **56,** 563–572
60. Treisman, R. (1985) *Cell* **42,** 889–902
61. Edwards, D. R., and Mahadevan, L. C. (1992) *EMBO J.* **11,** 2415–2424
62. Franco, A. R., Gee, M. A., and Guilfoyle, T. J. (1990) *J. Biol. Chem.* **265,** 15845–15849
63. Chen, J., and Varner, J. E. (1985) *EMBO J.* **4,** 2145–2151
64. Ecker, J. R., and Davis, R. W. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5202–5206
65. Smale, S. T., and Baltimore, D. (1989) *Cell* **57,** 103–113
66. Blackshear, P. J. (1993) *J. Biol. Chem.* **268,** 1501–1504
67. Giuliano, G., Pichersky, E., Malik, V. S., Timko, M. P., Scolnik, P. A., and Cashmore, A. R. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7089–7093
68. Moore, D. D., Marks, A. R., Buckley, D. I., Kapler, G., Payvar, F., and Goodman, H. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 699–702
69. Mathis, J. M., Houser, W. H., Bresnick, E., Cidlowski, J. A., Hines, R. N., Prough, R. A., and Simpson, E. R. (1989) *Arch. Biochem. Biophys.* **269,** 93–105
70. Sap, J., de Magistris, L., Stunnenberg, H., and Vennstrom, B. (1990) *EMBO J.* **9,** 887–896
71. Berkowitz, E. A., and Evans, M. I. (1992) *J. Biol. Chem.* **267,** 7134–7138
72. Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990) *Science* **249,** 1266–1272
73. Rogers, J. C., and Rogers, S. W. (1992) *Plant Cell* **4,** 1443–1451
74. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382
75. Anderson, D. J., and Blobel, G. (1983) *Methods Enzymol.* **96,** 111–120