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The *cis*-Acting Gibberellin Response Complex in High-pI α-Amylase Gene Promoters[1]

Requirement of a Coupling Element for High-Level Transcription

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In cereal α-amylase gene promoters the *cis*-acting gibberellin response element (GARE) is required for increased transcription in the presence of gibberellin. In low-isoelectric point (pI) α-amylase gene promoters a second type of *cis* element, termed a coupling element, must also be present in a specific position near the GARE; otherwise, the level of transcription in the presence of gibberellin is only a few percent of maximum. The coupling element may help determine where and when in development high-level, hormonally regulated transcription will occur. Such coupling elements have not yet been shown to be necessary for high-level transcription from high-pI α-amylase gene promoters. Here we use quantitative transient expression assays to show that a high-pI promoter truncated to −300 is a weak promoter due to the absence of a functional coupling element in the vicinity of the GARE. Gibberellin-induced transcription increases substantially when coupling element function is provided, either by appending upstream regions normally attached to the promoter or by inserting a defined coupling element from a low-pI promoter. Thus, in a second type of gibberellin-regulated promoter coupling element function was found to be crucial for hormone regulation to result in high-level transcription.

Cereal α-amylase genes are a primary model for the study of hormone-regulated transcription in plants. This model offers an understanding of not only how a hormone signaling pathway ultimately interacts with a short nucleotide promoter sequence to affect transcription, but also how other *cis*-acting DNA sequences interact with the hormone response element to direct high-level, hormone-regulated transcription in specific cells.

In barley (*Hordeum vulgare* L.), most α-amylase genes are expressed in aleurone cells but not in other tissues (Jacobsen et al., 1986). In the aleurone tissue, GA_3 increases their transcription, whereas ABA prevents this effect; however, one α-amylase gene that is expressed at low levels in barley leaf tissue does not respond in the same way to GA and ABA (Jacobsen et al., 1986). Those two phytohormones may be considered to be almost ubiquitously present in other tissues, where they also affect transcription of many other genes (Jones, 1973; Skriver and Mundy, 1990).

In aleurone cells, both phytohormones act on transcription through the same short nucleotide sequence in α-amylase gene promoters, the GARE (Skriver et al., 1991). The GARE sequence, UTAACAUANTCYGG (where U = purine, Y = pyrimidine, and N = any nucleotide), is highly conserved among different α-amylase gene promoters (Huang et al., 1990; Skriver et al., 1991; Rogers and Rogers, 1992). If the same intracellular pathways are used in all cells for signaling the presence of GA or ABA (an hypothesis that has not yet been proven), we must explain why a hormone affects transcription of a specific gene only in a specific cell type.

A precedent in animal cells may provide a useful model (Miner and Yamamoto, 1991; Pearce and Yamamoto, 1993). The glucocorticoid receptor binds its glucocorticosteroid hormone and then interacts with a conserved DNA sequence, the hormone response element. Other steroid hormones with different physiological roles, when bound to their specific receptor proteins, can also cause binding of those receptors to the same element. Therefore, binding of a hormone-receptor complex to the simple hormone response element is not sufficient to confer specific hormone-induced enhancement of transcription. Other factors that interact with adjacent elements provide specificity. For example, a composite glucocorticoid response element contains binding sites both for a receptor and for nonreceptor factors that determine which receptor can affect transcription (Miner and Yamamoto, 1991; Pearce and Yamamoto, 1993).

Studies of GARE function have shown some similarities to this composite response element model. In transient expression experiments, a GARE was able to confer proper hormonal regulation of transcription when attached to a minimal promoter, but only if multiple tandemly linked copies were used (Skriver et al., 1991). In contrast, within the context of an intact low-pI α-amylase promoter, the functionally defined GARE (Huttly et al., 1992; Lanahan et al., 1992; Rogers and Rogers, 1992) is a single-copy element. We showed that

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Abbreviations: ABRE, ABA response element; GARE, GA response element; GUS, β-glucuronidase; pI, isoelectric point.

a single GARE in this promoter is able to direct hormonally regulated transcription at a high level because it cooperates with other *cis*-acting elements (Lanahan et al., 1992; Rogers and Rogers, 1992).

The physically contiguous elements that together were termed a "gibberellin response complex" in the Amy32b low-pI α-amylase promoter (Lanahan et al., 1992; Rogers et al., 1992) are presented in Figure 1. As assessed by quantitative transient expression assays, the two most important elements were the GARE and an element termed O2S; mutation or deletion of either resulted in lower GA_3-induced transcription, to a level representing a few percent of that obtained with the intact promoter (Lanahan et al., 1992; Rogers and Rogers, 1992). Three other functionally defined elements contributed to high-level transcription, but mutation or deletion of these had smaller effects on transcription. Mutation of the sequence TATCCATGCAGTG (where the underlined sequence was replaced with TCTAGA in construct X7, Fig. 1), or mutation of the sequence CCTTTT (where the sequence was replaced with TCTAGA in construct X6, Fig. 1) lowered expression to about 20% of control levels (Lanahan et al., 1992). These two sequences, TATCCAT/C and CCTTTT, are also highly conserved among different α-amylase promoters, and mutation of TATCCAC in a barley high-pI promoter also lowered expression to about 20% of maximum (Gubler and Jacobsen, 1992). In our studies, deletion of a region encompassing "box 5" (Fig. 1) lowered expression to about 15% of maximum (Lanahan et al., 1992). The functionally important sequences in that region were identified in a low-pI wheat α-amylase gene promoter by footprinting with nuclear proteins from oat aleurone protoplasts (Rushton et al., 1992) and by functional analyses of deletions (Huttly et al., 1992).

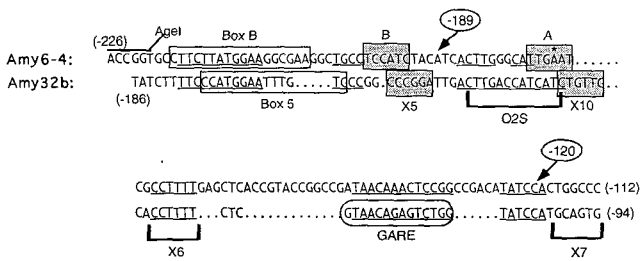

Figure 1. Comparison of promoter sequences from the Amy6-4 high-pI and the Amy32b low-pI α-amylase genes. The sequence of Amy6-4 from −226 to −112 is compared with that of Amy32b from −186 to −94. Underlined are nucleotides in Amy32b known to represent functional *cis*-acting elements and corresponding identical nucleotides in Amy6-4. In Amy32b these are identified as box 5 (Huttly et al., 1992; Rushton et al., 1992), O2S, GARE, CCTTTT as defined by the X6 mutation, and TATCCATGGAGTG as defined by the X7 mutation (Lanahan et al., 1992). Box B in Amy6-4 represents nucleotides footprinted by oat aleurone nuclear proteins (Rushton et al., 1992). Arrows from the circled numbers point to nucleotides −189 and −120; these delimit the sequence studied by Skriver et al. (1991). The asterisk above the A at −174 in Amy6-4 represents the 5′ end of the promoter fragment studied by Gubler and Jacobsen (1992). The shadowed boxes represent the sites of TCTAGA mutations inserted into Amy32b (X5 and X10, Lanahan et al., 1992) and into Amy6-4 (construct A and construct B, this work), respectively.

Because the GARE and O2S elements were most important for controlling the level of transcription in our promoter, we investigated the requirements for their functional interaction. We found that they functioned only when positioned in one orientation with respect to each other and with respect to the TATA box, and when the distance between them was relatively short (Rogers and Rogers, 1992). We created a promoter where transcription was increased in the presence of ABA and unaffected by GA simply by substituting an 11-nucleotide ABRE (Skriver et al., 1991) for the GARE; function of this ABRE was also found to require the presence of O2S (Rogers and Rogers, 1992). Those studies led us to conclude that both the GARE and the ABRE elements required the presence of a second element for high-level, hormone-regulated transcription. We termed the second element a "coupling element" to denote the following specific characteristics.

First, neither the hormone response element nor the coupling element alone caused an increase in transcription above a low level, indicating that neither element was capable of acting simply as a quantitative transcriptional enhancer in cooperation with other promoter elements. Second, the interaction between the hormone response element and coupling element had stringent positional and spatial requirements. This finding indicated that the coupling element was essential for assembly of factors that interacted with the hormone response element, resulting in hormone enhancement of transcription. In those studies, O2S served as the coupling element, but it is possible that other sequences also could serve that function. We speculated that the nature of the coupling element could determine cell type and developmental specificity of hormone-regulated transcription (Rogers and Rogers, 1992; Rogers et al., 1992).

From this perspective, results reported from functional analyses of deletions within a barley high-pI α-amylase gene promoter (Jacobsen and Close, 1991; Gubler and Jacobsen, 1992) were surprising. Those authors found that deletions from the 5′ end of a 2050-nucleotide promoter/upstream fragment had little effect on the level of transcription until they progressed within −174 (Jacobsen and Close, 1991; Gubler and Jacobsen, 1992), a position just 5′ to the CCTTTT element (Fig. 1). They proposed that essentially the GARE and TATCCAC elements alone were sufficient for high-level, hormone-regulated expression (Gubler and Jacobsen, 1992). Within −335 their promoter is identical in sequence to the Amy6-4 promoter (Khursheed and Rogers, 1988) used in the studies of Skriver et al. (1991), and Skriver et al. (1991) found that one copy of the GARE (albeit without the TATCCAC) was not sufficient to confer detectable hormone-regulated transcription on a minimal promoter in a similar protoplast transient expression system.

To explain apparent differences in results from experiments using the two different types of promoters, low pI versus high pI, we initiated the experiments described here. We provide quantitative analyses of interactions between the GARE and other *cis*-acting elements upstream from the GARE. Our results indicate that the GARE in high-pI α-amylase gene promoters has a similar requirement for coupling element function to enable high-level, hormone-regulated transcription.

MATERIALS AND METHODS

Transient Expression Assays

These assays used a barley (*Hordeum vulgare* L.) α-amylase/GUS reporter construct and an oat ubiquitin/luciferase internal standard construct that were bombarded together into intact barley aleurone layers as previously described (Lanahan et al., 1992; Rogers and Rogers, 1992). After 40 to 44 h, the bombarded half grains in sets of three were ground with a chilled mortar and pestle in 0.6 mL of ice-cold grinding buffer (Lanahan et al., 1992). After centrifugation at 10,000 *g* for 5 min, 100 μL of the supernatant was assayed for luciferase activity, and 50 μL, diluted into a final volume of 0.125 mL, was assayed for GUS activity (Lanahan et al., 1992). Specifically, for the GUS assay, 25-μL aliquots were removed at zero time and at subsequent intervals up to 4 h and diluted into 2 mL of 0.2 M $NaCO_3$, and the resulting fluorescence was measured in a Sequoia-Turner model 450 fluorometer where 0.1 μM 5-methylumbelliferone gave a reading of 1000 units. The direct reading from the fluorometer for a 4-h time point under conditions where the GUS activity was linear, minus the zero-time value, was then corrected to be expressed as the total number of fluorescent units that would be generated in 4 h from an aliquot of extract that contained 35,000 relative light units of luciferase activity. This number was termed "GUS units." Statistical comparisons of results were made using Student's *t* test.

Recombinant Constructs

Construction of JR248 and its derivative ML022, in which the promoter/upstream sequence was truncated to −331, have been described (Lanahan et al., 1992). *JR249*: A *Bam*HI site was introduced into the sequence of the high-pI α-amylase gene, Amy6-4 (Khursheed and Rogers, 1988), immediately in front of the ATG translation initiation codon with a synthetic oligonucleotide and PCR amplification as previously described (Rogers and Rogers, 1992). Using the same approach, the sequence of the first intron and nucleotides flanking the 5′ and 3′ splice sites was introduced into the sequence immediately upstream from the *Bam*HI site. (The exact sequence is available from the authors upon request.) The approximately 1.8-kb *Hin*dIII-*Bam*HI fragment containing the promoter, transcription start site, 5′ untranslated sequence, and first intron was then substituted for the corresponding fragment in JR248. This gave a construct with the GUS coding sequence followed by the Amy32b poly(A) addition sequence under transcriptional control of the Amy6-4 promoter. *JR265*: The transcription initiation site in the high-pI α-amylase gene Amy46 (Khursheed and Rogers, 1988) was mutated to create an *Nsi*I site as found in Amy6-4. The 0.4-kb *Xho*I-*Nsi*I fragment from Amy46 was then substituted for the 0.64-kb, similarly positioned fragment in JR249. *JR366*: Using PCR, a *Hin*dIII site was inserted at −301 in the JR249 promoter. *Construct A (JR368)*: Using single-strand mutagenesis as previously described (Lanahan et al., 1992), an *Xba*I site was inserted at −177 in JR366 (Fig. 1). *Construct B (JR367)*: Using single-strand mutagenesis, an *Xba*I site was inserted at −193 in JR366 (Fig. 1). *Construct C (JR369)*: The *Hin*dIII-*Xba*I fragment from construct B was recombined with the *Xba*I-*Bam*HI fragment of construct A. *Construct D (JR394)*: The *Age*I-*Xba*I fragment from construct B was removed, the ends were polished with Klenow fragment of DNA polymerase I, and the blunt ends were religated. *Construct E (JR393)*: The *Hin*dIII-*Age*I fragment from construct B was removed, and the ends were polished and ligated as for construct D. *Construct F (JR395)*: The *Hin*dIII-*Age*I fragment from JR249 was inserted into the *Hin*dIII-*Age*I interval of construct B. *Construct G (JR378)*: Using PCR, an intact O2S sequence was substituted for the corresponding nucleotides in JR366; the exact sequence is presented in Figure 5, below. *Construct H (JR396)*: The *Hin*dIII-*Age*I interval from construct G was deleted as in construct E. *Construct I (JR370)*: The *Hin*dIII-*Xba*I fragment from construct X10 (Lanahan et al., 1992) was substituted for the corresponding fragment in construct A. *Construct J (JR375)*: The *Hin*dIII-*Xba*I fragment from construct X5 (Lanahan et al., 1992) was substituted for the corresponding fragment in construct B. *Construct K (JR401)*: An *Age*I site was introduced by PCR at position −184 of the low-pI promoter sequence in construct I, and the resulting *Age*I-*Xba*I fragment was substituted into the corresponding position of construct G. The resulting sequence is shown in Figure 5, below. *Construct L (JR402)*: An *Nsi*I site was inserted at position +1 in ML022 by PCR; the *Hin*dIII-*Nsi*I promoter fragment was then substituted for the corresponding fragment in JR366.

Sequence Alignment Comparisons

The sequence of the high-pI α-amylase genomic clone studied by Jacobsen and Close (1991), termed BLYAMY1G in GenBank, was aligned to the sequence of Amy6-4 (Khursheed and Rogers, 1988) using the Bestfit alignment program in the University of Wisconsin Genetics Computer Group Software Package carried in the Department of Biology VAX computer.

RESULTS

Quantitative Comparisons of Transcription from Different Low-pI and High-pI α-Amylase Promoters

The high-pI α-amylase promoter used in the experiments of Jacobsen and Close (1991) is identical in sequence from the transcription initiation site up to −335 when compared with the promoter from our high-pI α-amylase gene, Amy6-4 (Khursheed and Rogers, 1988); upstream from this position the two sequences show no similarity (data not presented). Jacobsen and Close (1991) found little effect on transcription when the 5′ end of their promoter was deleted from −2050 to −174. Therefore, we could construct a promoter identical to that used in those experiments (Jacobsen and Close, 1991; Gubler and Jacobsen, 1992) by truncating our Amy6-4 promoter down to within −335, but first had to establish if our promoter contained elements upstream of that position that affected its function.

We first determined the relative strengths of different α-amylase promoters in our particle-bombardment system using intact aleurone layers (see "Materials and Methods"). In Figure 2 results are presented from four different promoter constructs: JR248 contains 1.6 kb of the Amy32b low-pI α-

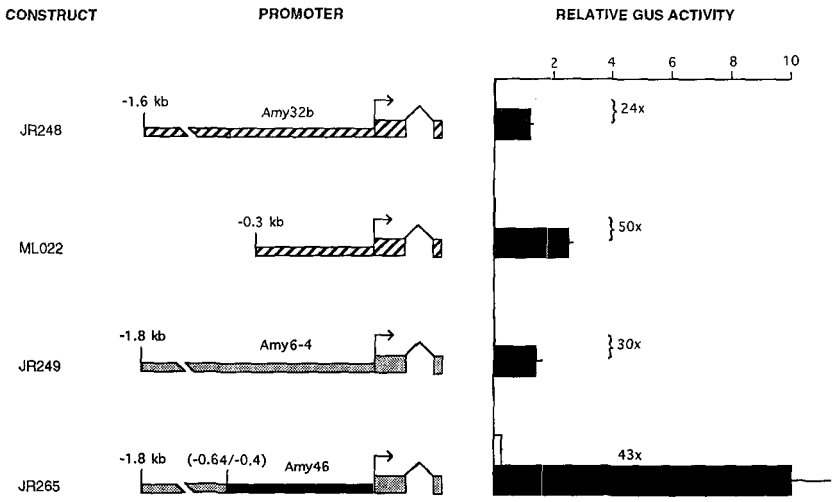

Figure 2. Levels of transcription obtained from different α-amylase promoter constructs. All constructs shown contained the same GUS coding sequence/Amy32b poly(A) addition sequence components described by Lanahan et al. (1992). A diagram for each construct (shown under Promoter) indicates the nature and length of the promoter/upstream sequences. The narrow diagonally striped rectangle indicates promoter/upstream sequences from the low-pI α-amylase gene, Amy32b; numbers at the left end indicate distance upstream from the transcription initiation site. Similarly, the narrow stippled rectangle indicates promoter/upstream sequences from the high-pI α-amylase gene, Amy6-4. In JR265, the black narrow rectangle indicates sequences substituted from the high-pI α-amylase gene, Amy46 (Khursheed and Rogers, 1988). In each diagram, the arrow indicates the transcription initiation site, the thick striped or stippled rectangles indicate 5′ untranslated sequences, and the peaked line connecting the rectangles indicates the intron sequence from Amy32b (connecting striped boxes) or Amy6-4 (connecting stippled boxes). Relative GUS Activity compares the level of expression of each construct with that obtained for JR265, which was arbitrarily given a value of 10. The open bars (not visible for JR248, ML022, and JR249) represent expression in the absence of hormone, and the dark stippled bars represent expression in the presence of 10^{-6} M GA_3. Lines at the ends of the bars represent the SE. The numbers after the brackets to the right of each pair of bars (e.g. 24x) represent the fold increase in expression obtained in the presence of GA_3.

amylase promoter/upstream sequences (Whittier et al., 1987); ML022 represents the same promoter truncated to −331 (Lanahan et al., 1992); JR249 contains 1.8 kb of the Amy6-4 high-pI α-amylase promoter (Khursheed and Rogers, 1988); and JR265 represents that construct where the interval from the transcription initiation site to −640 was substituted with the corresponding fragment to −400 from the Amy46 high-pI α-amylase promoter (Khursheed and Rogers, 1988). In each construct, the reporter gene was *Escherichia coli* GUS (Lanahan et al., 1992). It can be seen that the relative transcriptional strength of the promoters in response to GA_3 varied over a 10-fold range, with JR248 being the weakest and JR265 the strongest. All responded to the presence of GA, with the GA-induced increase in transcription ranging from 24-fold for JR248 to 50-fold for ML022. To put these data into perspective, when directly compared, expression of the cauliflower mosaic virus 35S promoter-maize shrunken intron-GUS construct (gift of Dr. L.C. Hannah, University of Florida, Gainesville, FL) was 60% of that obtained with ML022 in the presence of GA_3 (data not presented).

Deletion Analysis of the Amy6-4 Promoter

The sequence of the Amy6-4 promoter between −226 and −112 is presented in Figure 1, where it is compared with the corresponding region from the low-pI α-amylase promoter, Amy32b. It is important to note the portions of this promoter studied by other authors. The fragment studied by Skriver et al. (1991) in their characterization of the GARE is indicated by the arrows at −189 and −120. The position of the −174 deletion used by Gubler and Jacobsen (1992) is indicated by an asterisk. Functionally defined sequences in Amy32b that are conserved in Amy6-4 are underlined, e.g. the CCTTTT, GARE, and TATCCAC/T elements. Upstream from the CCTTTT, however, the two sequences appear to be more divergent. There appears to be only moderate conservation of sequence between the two within the regions corresponding to the important O2S element and the box 5 element in Amy32b. Two other features of the Amy6-4 sequence are important to note. Rushton et al. (1992) identified two sequences in the promoter of their wheat high-pI gene that footprinted with nuclear proteins from oat aleurone protoplasts. One, called box A, was just upstream of, and included the 5′ end of, the *AgeI* site (Fig. 1). The other, called box B, is indicated in Figure 1 and overlaps part of the sequence corresponding to the functionally defined box 5 region in the low-pI genes (Huttly et al., 1992; Rushton et al., 1992).

In each transient expression experiment, the level of expression of the low-pI promoter construct ML022 (Lanahan et al., 1992) was also assayed, and results for each of the high-pI constructs were expressed as a percentage of the level obtained with ML022 so that constructs tested in different experiments could be compared. We first compared transcription from the intact 1.8-kb Amy6-4 promoter/upstream se-

quence (JR249) to that from a version (JR366) truncated at −301 (Fig. 3). JR249 was expressed at a level 42% of that obtained with ML022 (consistent with results in Fig. 2). JR366, however, gave only a 10-fold increase in the presence of GA_3, to a level representing only 10% of that for ML022. This result suggested that sequences between −1.8 and −301 kb were able to increase transcription 4-fold from this promoter. This was confirmed by substituting the 1.6-kb *Hin*dIII-*Age*I fragment from JR249 (Fig. 3, A) for the corresponding fragment in a version of the truncated promoter that contained an *Xba*I linker mutation (Fig. 3, B) to give the construct shown in Figure 3, F. The expression of construct F was indistinguishable from that of JR249. Therefore, in the absence of these "enhancing" upstream sequences in Amy6-4, which may not be found in the promoter used by Jacobsen and Close (1991) because the two promoters are divergent in that region, the truncated promoter was hormonally regulated but transcribed at a low level.

We then investigated whether sequences between −301 and the CCTTTT element affected transcription from the truncated promoter. We first introduced 6-nucleotide mutations to encode sites for the restriction enzyme *Xba*I into the promoter at −177 (Fig. 3, A) and −193 (Fig. 3, B). These positions were chosen because they approximated the positions of the X10 and X5 mutations, respectively, in the Amy32b promoter (Fig. 1). These flanked the region corresponding to the expected position of an O2S element (indicated by ? in Fig. 3). It can be seen that neither mutation affected transcription from the promoter (Fig. 3, A and B, compared with JR366). When the two *Xba*I sites were recombined to delete the putative O2S between −193 and −177, transcription from the resulting construct (Fig. 3, C) was not significantly different from that of the parent constructs (Fig. 3, A and B). There was a trend for expression from the −193/−177 deletion construct C to be higher than that obtained with the basic truncated promoter, JR366, but when the two were compared in the same experiment the difference was not statistically significant ($P > 0.05$). These results demonstrated that the Amy6-4 promoter does not have a functional O2S element that increases transcription in the presence of the GARE at a position comparable to the low-pI promoter.

We then tested the effects of deleting regions containing sequences that interacted with aleurone cell nuclear proteins (Rushton et al., 1992). When the −226 to −193 region containing box B (Fig. 1) was deleted, transcription was not affected (Fig. 3, D versus B). In contrast, however, when the −301 to −226 region containing box A (Rushton et al., 1992) was deleted, transcription decreased significantly (Fig. 3, E versus B, 5% versus 12%, $P < 0.025$). This result demonstrated that sequences within the −301 to −226 interval, possibly box A, positively affected transcription from the truncated promoter. It emphasizes that the high-pI promoter truncated to −226 is weak, with a GA_3-induced level of expression only 5% of that obtained with the low-pI promoter

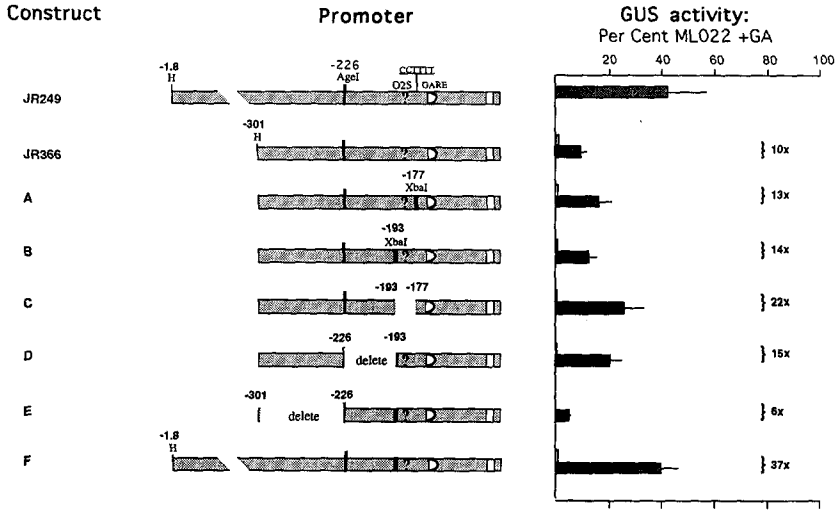

Figure 3. Analysis of deletions within the high-pI promoter. Under the heading, Construct, are the names by which the constructs are identified. Under the heading, Promoter, are diagrams of promoter constructs used in each of the experiments; the right ends of these diagrams represent the transcription initiation sites. The open rectangle represents the TATA box at −29. The position of the GARE is indicated by an open half circle. Relative to that is indicated the position of the CCTTTT (above JR249), and the position of a putative O2S element is indicated by a question mark. The position of the *Age*I site at −226 is indicated by a thick vertical line. The positions of *Xba*I sites introduced by mutagenesis are indicated by small black rectangles within the stippled boxes. Deletions are indicated by gaps in the stippled boxes, and the extents of those deletions are marked by nucleotide positions above each construct. The sloping gaps in the stippled boxes for JR249 and F indicate that the diagrams are not drawn to scale upstream of −301. The 5′ *Hin*dIII site for each construct is indicated by H above its end. GUS activity obtained for each construct is expressed as a percentage of that obtained with ML022 analyzed in the presence of GA_3 in the same experiment (see "Materials and Methods"). Bars representing GUS activity are as in Figure 2. In this set of experiments JR249 was not analyzed in the absence of hormone.

construct, ML022. It is important to note that the level of expression obtained with the −226 truncation, construct E, is similar to that obtained when the O2S was deleted or mutated within the ML022 low-pI promoter (Lanahan et al., 1992; Rogers and Rogers, 1992).

Addition of a Functional Coupling Element to the High-pI Promoter

The above results suggested that the high-pI promoter truncated to −226 was weak because it lacked a functional box 5 sequence, a functional O2S, or both. (Results from the low-pI promoter system indicated that the function of the box 5 element probably could not be assessed in the absence of a functional O2S, because loss of the latter caused such a profound decrease in transcription [Lanahan et al., 1992].) If this hypothesis were correct, substitution of functional box 5 and O2S elements from the low-pI promoter would be expected to increase transcription substantially from the high-pI promoter.

We first substituted an intact O2S sequence into the high-pI promoter truncated at −301 (Fig. 4, G) or at −226 (Fig. 4, H); the precise sequence is shown in Figure 5. This substitution had little effect on transcription. However, when the portion of Amy32b from −137 to −331, which contains the O2S and box 5 elements (Fig. 1), was substituted for the portion of the high-pI promoter upstream from −177, GA_3-induced transcription increased to a level not statistically different from that obtained with the intact ML022 low-pI construct (Fig. 4, I). This result demonstrated that the CCTTTT-GARE-TATCCAC portion of the high-pI promoter could interact properly with coupling element function provided by the low-pI promoter fragment to give high-level transcription. The expression levels obtained with constructs G and H (Fig. 4) were 13 to 16% of that from construct I, a finding consistent with the possibility that the former two

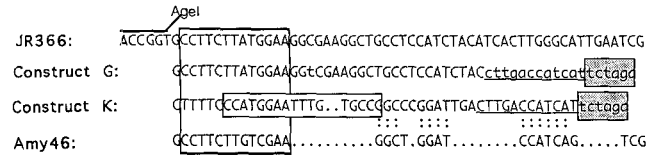

Figure 5. Comparison of the box 5-O2S regions in different promoter constructs. The sequence of the Amy6-4 high-pI promoter in construct JR366 from −226 to −170 is shown on the top so that other sequences can be compared with it. Construct G represents that sequence into which an *Xba*I site (lowercase letters in stippled box) and an O2S sequence (lowercase letters, underlined) have been substituted. The single lowercase t in that sequence represents an error introduced during the PCR process. Construct K represents replacement of the sequence between the *Age*I and *Xba*I sites with a sequence from Amy32b that contains the functional box 5 (open box) and O2S (underlined) sequences. Amy46 presents the corresponding sequence from that high-pI α-amylase gene; gaps in that sequence needed for proper alignment are represented by dots. Sequence identity between Amy32b and Amy46 is indicated by two dots. The large open box enclosing 13 nucleotides from each of the sequences represents a conserved box B motif (see Fig. 1).

constructs were expressed at lower levels because each lacked a functional box 5 sequence. (Deletion of the box 5 function in the low-pI construct lowered expression to approximately 15% [Lanahan et al., 1992].)

The relative contributions of box 5 and O2S sequences were clarified by making different low-pI/high-pI promoter chimeras. When the −159 to −331 portion of the low-pI promoter containing the box 5 sequences was substituted for the portion of the high-pI promoter upstream from −190, GA_3-induced transcription was only 7% of that obtained with the ML022 low-pI promoter (Fig. 4, J). This result is what would be expected from deleting or mutating a functional O2S out of construct I (Fig. 4). To establish that only box 5

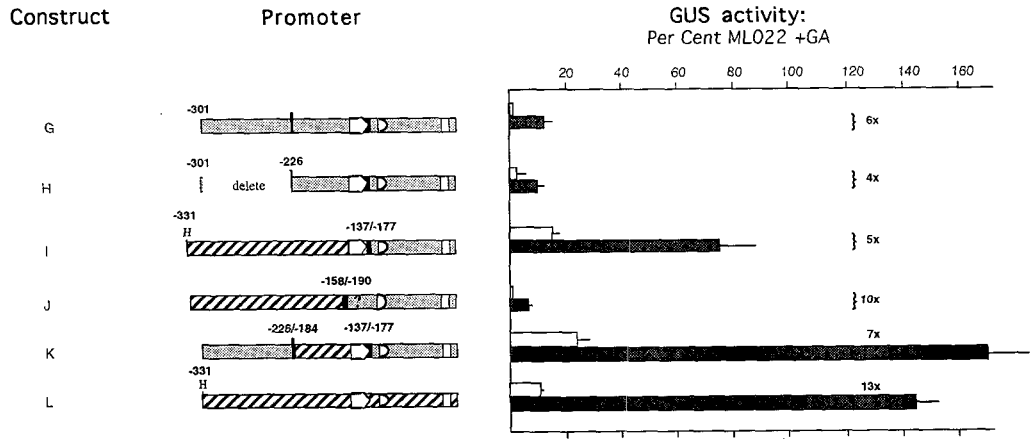

Figure 4. Transcriptional effects resulting from the introduction of low-pI promoter sequences into portions of the JR366 high-pI promoter. Construct designation, diagrams of promoter constructs, and manner of expressing GUS activity are as in Figure 3. Additionally, for promoter construct diagrams, the presence of an intact O2S element is indicated by an open pentagon. Striped regions of the construct diagrams represent sequences from the low-pI promoter, Amy32b, that were substituted for high-pI promoter sequences. Construct L differs from the low-pI promoter construct, ML022, in that a high-pI 5′ untranslated sequence plus intron are present in the transcript from construct L and a low-pI 5′ untranslated sequence plus intron are present in the transcript from ML022.

and O2S sequences in the low-pI promoter fragment used in construct I were responsible for giving the level of expression observed with that construct, we substituted the −184 to −137 sequences from the low-pI promoter into the high-pI promoter −226 to −177 interval (Fig. 4, K; precise sequence shown in Fig. 5). This construct gave a level of GA_3-induced expression 166% of that obtained with ML022, a result that confirms the importance of the box 5 and O2S sequences in coupling the GA_3 effect to high-level transcription. This represents 14- and 16-fold increases over the levels of expression obtained with constructs G (Fig. 4) and JR366 (Fig. 3), respectively.

As a final control, we had to consider the fact that the RNA transcript made from ML022 had a low-pI 5′ untranslated sequence and intron, whereas constructs based on JR249 had a high-pI 5′ untranslated sequence and intron. It was necessary to exclude the possibility that these differences might somehow affect the levels of expression measured in the preceding experiments. Therefore, the effects of the two different 5′ untranslated sequences/introns on levels of expression measured in this assay were tested by placing the ML022 low-pI promoter onto the high-pI 5′ untranslated sequence/intron construct (Fig. 4, L). This construct gave a level of expression 140% of that obtained with ML022, demonstrating that the differences in levels of expression between low-pI and high-pI constructs measured in Figures 2, 3, and 4 were due to transcription and did not reflect differences in rates of RNA splicing, RNA stability, or mRNA translation efficiency.

DISCUSSION

Barley aleurone cells synthesize and secrete large amounts of α-amylase in response to GA_3 (Yomo and Varner, 1971). This results from increased mRNA levels from two α-amylase multigene families. The low-pI gene family has three members; two of these are transcribed at high levels. The high-pI family has seven or eight members (Khursheed and Rogers, 1988; Rogers and Khursheed, 1989). In isolated aleurone layers, the GA_3-induced, steady-state level for mRNA from all high-pI genes is approximately the same as that for mRNA from the two low-pI genes. However, it has not been possible to determine which high-pI genes are expressed at the highest levels because the sequences of 5′ untranslated regions in seven of the approximately eight different mRNAs are very similar as determined by primer extension/sequencing (Rogers, 1985; Chandler and Huiet, 1991) and by S1 nuclease protection assays (Khursheed and Rogers, 1988). Indeed, those genes are so similar that their promoter regions cross-hybridized at high stringency (Khursheed and Rogers, 1988). The only one of the eight high-pI genes that could be distinguished by those methods was Amy46 (Khursheed and Rogers, 1988); it has substantial divergence within its 5′ untranslated region, and its promoter differs so that an Amy6-4 promoter probe did not hybridize to the Amy46 promoter at high stringency (Khursheed and Rogers, 1988).

As a result of the striking sequence conservation among these approximately seven barley high-pI genes and the resulting difficulty of assigning steady-state mRNA levels to any one, it would be difficult to predict if the promoter from any one genomic clone would be likely to be strong or weak. This difficulty explains one reason for experiments performed in this paper. From 3′-specific probes used in S1 nuclease protection assays, we estimated that mRNA from the Amy6-4 gene provided a major portion of the total high-pI mRNA (Khursheed and Rogers, 1989) and therefore guessed that the Amy6-4 promoter would be relatively strong. This prediction was confirmed by finding that the Amy6-4 promoter in JR249 was approximately as strong as the low-pI promoter in JR248 (Fig. 2). Consistent with what is known about the similarity of different members of the high-pI gene family, the promoter and 5′ untranslated sequence of the gene used by Jacobsen and Close (1991) and Gubler and Jacobsen (1992) are identical to Amy6-4 beginning at position −335. Those authors found that the level of GA-induced transcription from a construct extending upstream to −2050 was similar to that obtained from the same promoter truncated to −174. We created a similar construct by truncating the Amy6-4 promoter to −301. That truncated high-pI promoter was much weaker than the full-length (−1.8 kb) promoter from Amy6-4; the difference between the two was attributable to sequences in Amy6-4 between −1.8 and −0.33 kb that increased transcription 4- to 5-fold (Fig. 3). At present we do not know if they provide only a general transcriptional enhancing function, which would interact with many different types of promoters, or if they represent another type of coupling element where their function would be dependent on the presence of the GARE. It will be of some interest to identify those sequences in the future and to characterize their interaction with the GARE.

The −301 high-pI promoter was weak because it lacked a functional coupling element. When that function was provided by substituting box 5 + O2S sequences into a position upstream from the GARE, transcription from the promoter increased 16-fold, to a level comparable to that of the low-pI promoter in ML022. The fact that the baseline (no hormone) level of transcription increased in the chimeric low-pI/high-pI constructs (e.g. Fig. 4, I and K) over what would be seen with either an intact low-pI or high-pI promoter indicates that the fine control of GARE function may have been disturbed. This may result from subtle differences in interactions of other elements in the two different types of promoter. However, it does not negate the central point of this paper: a GARE plus TATCCAC/T alone in either type of promoter permits only low-level transcription, and high-level expression requires the action of what we have termed a coupling element.

Substitution of the Amy46 promoter sequence from its transcription initiation site upstream to −400 into JR249 to give construct JR265 resulted in a promoter that was 10 times stronger (Fig. 2). The sequences of the Amy46 and Amy6-4 promoters differ substantially, primarily upstream from the CCTTTT element (Khursheed and Rogers, 1988), and it is reasonable to speculate that elements responsible for the strength of JR265 are found in the divergent region. The strength of the promoter containing Amy46 sequences suggests that other mechanisms, such as RNA processing or mRNA stability rather than transcription, may account for the low level of Amy46 mRNA in GA_3-treated aleurone layers (Khursheed and Rogers, 1988). In Figure 5 the sequence of

Amy46 in the region corresponding to the box 5-O2S region is compared with that of the low-pI promoter sequence (construct K) and with that of the Amy6-4 high-pI sequence (JR366). The Amy46 sequence has substantial deletions (indicated by gaps with dots, Fig. 5), but the remaining sequence appears to be more similar to the low-pI sequence than to Amy6-4. It will be important in the future to map elements in the Amy46 promoter that couple hormone regulation to high-level transcription. By characterizing such elements in different promoters, we will be able to understand in more detail how they participate in cell- or tissue-specific regulation of transcription and whether their interactions are limited to any specific hormone response element.

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