Elements of the Maize A7 Promoter Required for Transactivation by the Anthocyanin B/C1 or Phlobaphene P Regulatory Genes

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The extensive genetic and molecular characterization of the flavonoid pathway's structural and regulatory genes has provided some of the most detailed knowledge of gene interactions in plants. In maize flavonoid biosynthesis, the A7 gene is independently regulated in the anthocyanin and phlobaphene pathways. Anthocyanin production requires the expression of the C1 or PI and R or B regulatory genes, whereas phlobaphene production requires only the P regulatory gene. By deletion analysis of the A7 promoter, we show that the sequences between -123 and -88 are critical for activation by anthocyanin and phlobaphene regulatory genes. Linker-scanner mutations indicated that the -123 to -100 region is more important for transactivation by the P protein. The -98 to -88 region is more important for B/C1 transactivation and shows a strong homology with the region of the Bz1 anthocyanin structural gene promoter shown to be activated by B/C1 and not by P. We identified a 14-bp consensus sequence that is also present in the promoters of three other genes in the anthocyanin pathway, and we propose a model for how the flavonoid regulatory proteins interact with the promoters of the structural genes.

INTRODUCTION

The extensive genetic and molecular characterization of flavonoid pigment production in maize, Antirrhinum, and petunia has provided some of the most detailed knowledge of gene interactions in plants. Most of the structural and regulatory genes have been isolated, and extensive genetic data exist on their developmental and environmental regulation, as well as their often complex allelic interactions and expression patterns (for review, see Dooner et al., 1991). In maize, the flavonoid pathway produces both the anthocyanins, which are located in most parts of the plant, and the phlobaphenes, which are limited primarily to the seed pericarp, cob, and floral structures. The two pigment pathways share the initial biosynthetic steps encoded by the genes C2 (chalcone synthase; Wienand et al., 1986), CHI (chalcone isomerase; Grotewold and Peterson, 1994), and A7 (NADPH-dependent flavonol reductase; Schwarz-Sommer et al., 1987). However, subsequent structural genes in the anthocyanin pathway, such as A2 (flavonol dehydroxylase; Menssen et al., 1990), Bronze-1 (Bz1, UDP glucose:flavonol-3-O-glucosyl transferase; Furttek et al., 1988), and Bronze-2 (Bz2; Nash et al., 1990), are not required in the phlobaphene pathway (Coe et al., 1986).

Four regulatory genes in two gene families control the expression of the anthocyanin biosynthetic structural genes. A member of each of the B/R and C1/PI gene families must be expressed for activation of the anthocyanin pathway. The B and R genes show extensive allelic diversity (Styles, 1970) and can substitute for each other genetically or when expressed from a constitutive cauliflower mosaic virus (CaMV) 35S promoter (Ludwig et al., 1989; Goff et al., 1990). The B/R proteins show sequence homology to the basic-helix-loop-helix (bHLH) DNA binding/dimerization domain found in MYC and MYOD proteins (Chandler et al., 1989; Ludwig et al., 1989). The C1 and PI genes show less genetic diversity and encode proteins with sequence homology with the DNA binding regions of the mammalian MYB protooncogene family of proteins (Paz-Ares et al., 1987; Cone et al., 1993).

Genetic studies have found P to be the only regulatory gene in the phlobaphene biosynthetic pathway of maize (Styles and Ceska, 1977). The P gene regulates the accumulation of the transcripts of the C2, CHI, and A7 genes in the pericarp but does not activate the other anthocyanin structural genes such as Bz1 (Grotewold et al., 1994). The P protein has homology with the DNA binding regions of MYB proteins, and this N-terminal region of the predicted P protein is 80% identical to the C1 protein (Grotewold et al., 1991). P protein produced in Escherichia coli binds to the consensus site ACCCTCCA in vitro, and this sequence appears to play a role in the inducibility of the A7 promoter in vivo (Grotewold et al., 1994).

Previously, a deletion analysis of the Bz1 promoter defined a cis-acting region that responds to regulation by B and C1...
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The experimentally defined region important for A1 promoter activity, a series of 5' deletions of the A1 promoter in their normal orientation were used in a transient expression assay system to define the critical regions required for B/C1 or P inducibility. Two regions containing myb-like sites were found to be important. The 5' site was more important for P induction and contained a P-like site, whereas both sites were important for B/C1 induction of the A1 promoter. Adjacent to these two myb-like sites was an area critical for B/C1 transactivation. When aligned with the critical region of the Bz7 promoter, a 15-bp region of the A7 promoter was identical at 11 nucleotides. A 14-nucleotide consensus sequence of this region can also be found in C2, A2, and Bz2 anthocyanin structural gene promoters. These results, together with earlier results on the functional domains of the regulatory proteins, are used as the basis of a model for how the flavonoid regulatory proteins interact with the promoters of the structural genes.

RESULTS

Expression of 5' Deleted A1 Promoter Constructs

The sequence of the A1 promoter from −130 to +1 contains several possible myb-like sites and two P-like sites (Figure 1A). The P protein has been demonstrated to bind to the P site at −60 (Grotewold et al., 1994). To define the 5' boundary of the region important for A1 promoter activity, a series of 5' deletions of the 1.4-kb A1 promoter (Schwarz-Sommer et al., 1987; Klein et al., 1989) was constructed. Each construct derived from A1-Luc (Figure 1B) contained a 5' deleted A1 promoter fragment (5' end points from −1400 to +10), the first intron from the Bz1 gene to increase the level of expression (Callis et al., 1987), the coding region from the firefly luciferase (Luc) gene (Ow et al., 1987), and the 3' polyadenylation region from A1.

LUC activity from the constructs was measured in transient gene expression assays following the introduction of the plasmids into Black Mexican Sweet (BMS) maize suspension cells by microprojectile bombardment. The BMS cells require the expression of both B and C1 to transactivate the A1 or Bz1
promoters and to synthesize anthocyanin pigments. The A7 promoter can also be transactivated by P alone (Grotewold et al., 1994). Thus, expression from the A1-Luc constructs was transactivated by cobombardment with either B and C1 plasmids or a P plasmid. A plasmid containing the chloramphenicol acetyltransferase (CAT) gene driven by the maize alcohol dehydrogenase 1 (Adh1) promoter (pAdh-CAT; Callis et al., 1987) served as an internal control for the efficiency of the bombardment and extraction (Klein et al., 1989). Expression from the A1-Luc plasmids was recorded as a ratio of LUC-to-CAT activity.

The activity of the 5′ promoter deletions relative to the activity of the full-length 1.4-kb A7 promoter is exhibited in Figure 2. Relative activities from the constructs with 5′ deletion end points between −600 and −123 were essentially equal to the full-length promoter. For the constructs with end points at −98 and −91, relative activities of 5 and 8% were observed. The relative activities of constructs with end points at −78, −34, and +10 were 2% or less. None of the 5′ deletions or the intact A7 promoter was transactivated by either B or C1 alone (data not shown) or expressed at significant levels in the absence of both B and C1 (Figure 2). These results suggest that the regions essential for the regulation of A7 by B and C1 or P lie within 123 bp of the start of transcription.

The binding of Escherichia coli–expressed C1 protein to the 3′ end of the A7 gene has been reported previously (Wienand et al., 1990). To exclude the possibility that A1-Luc expression was influenced by sequences from the 3′ end of A7, this region of A1-Luc was replaced with a fragment containing the 3′ polyadenylation region from the nopaline synthase gene. When cobombarded with B/C1 plasmids, LUC activity from this construct was equivalent to that from the plasmid with the A7 3′ end (data not shown). This result indicates that the 3′ end of the A7 gene does not contain an enhancer necessary for promoter activity.

Expression of 3′ Deleted A1 Promoter Constructs

To delineate the 3′ boundary of the regions responding to B/C1 and P regulation, it was necessary to distinguish between minimal core promoter sequences and B/C1- or P-responsive sequences. This was achieved by fusing 3′ deletions of the A7 promoter to a minimal Adh1 promoter (5′ end located at Adh1 nucleotide position −89; Figure 1B). Attaching the B/C1- and P-responsive regions of the A7 promoter upstream of the Adh1 core promoter should confer B/C1 and P inducibility to the Adh1 promoter because the Adh1 core promoter was shown previously to be inducible by B/C1 when fused to the Bz1 promoter (Roth et al., 1991).

The 3′ A7 promoter deletion constructs contained a fixed 5′ end point at −1400 with 3′ deletion end points at −30, −55, −88, −108, −118, and −128. The A7 3′ deletion–Adh1 promoter constructs were cobombarded into BMS cells, along with B and C1 or P expression vectors and pAdh-CAT. The LUC activity levels were measured relative to those of the Adh1 core promoter (Figure 3). In the absence of B/C1 or P, all of the 3′ deletion constructs expressed at the same level as the Adh1 core promoter. This indicates that there is no enhancement or repression of the A1-Adh1 core promoter fusions in the absence of B/C1 or P. However, in the presence of B/C1 or P, the A1 promoter fragments with 3′ boundaries at −30, −55, and −88 resulted in a five- to 10-fold increase in LUC activity of the Adh1 core promoter. The absolute amounts of LUC produced from these constructs were as high or higher than those produced from the intact A1 promoter in the A1-Luc construct. This indicates that the A1 regions of the fusion constructs are providing a strong enhancement of the minimal Adh1 core promoter.

Only a twofold increase in expression was observed for the A1-Adh1 constructs with 3′ end points at −108 and −118. Both the −128 end point and the minimal Adh1 core promoter alone showed no induction with either B/C1 or P. These data define the critical 3′ boundary of the B/C1- and P-responsive sequences of the A7 promoter to be at −88. For comparison, the inducibility was measured from the previously reported construct containing Bz1 sequences (−515 to −45) upstream of the Adh1 minimal core promoter (Roth et al., 1991). The Bz1 sequences increased the Adh1 minimal promoter activity approximately sevenfold when cobombarded with B and C1 (Figure 3). The coexpression of P did not increase the Bz1-

Figure 2. Transient Expression of 5′ Deletion Mutants of the 1.4-kb A1 Promoter.

Constructs with 5′ promoter deletions of A1-Luc were bombarded into BMS-EG5 cell suspensions along with plasmids expressing the B/C1 or P cDNAs and the Adh-CAT reference plasmid. The bars represent the relative LUC activity versus the amount from the intact 1.4-kb A1 promoter in A1-Luc with B/C1 (black columns), P (striped columns), or no transacting factor present (white columns; values not determined for the −91, −78, and +10 deletions). At least five independent bombardments were performed for each construct. Error bars represent the standard error of the mean.
Figure 3. Enhancer Assay of 3' Deletions of the A7 Promoter Fused 5' of the Adhl Core Promoter.

The indicated A7 promoter 3' deletion end points were fused to the -89 Adhl minimal promoter and bombarded into BMS-EG5 cell suspensions. The relative enhancement of activity was measured when cell suspensions were bombarded alone or with B/C1 or P. Shown for comparison is the value for the fusion of the 3' end of the Bz7 promoter at -45 (-45 Bz7) to the -89 Adhl minimal promoter. These values were compared to the expression from the -89 Adhl minimal promoter. The bars represent the activities with B/C1 (black columns), P (gray striped columns), or no transacting factor present (white-striped columns). Error bars represent the standard error of the mean.

Adhl minimal promoter expression (Figure 3). This was expected because the Bz7 promoter is not induced by P (Grotewold et al., 1994).

Site-Directed Mutations of the A7 Promoter

The results presented above indicate that the sequences responsible for the transactivation by B/C1 or P are located between -88 and -123 of the A7 promoter. To further define specific sequences necessary for regulation by B/C1 and P, linker-scanner mutations were constructed in the -128 to -59 region. Each mutation replaced 10 nucleotides of the A7 promoter with 10 nucleotides that included an Xhol site (Figure 4). Only a 10-bp region of the entire 1.4-kb A7 promoter sequence is changed in each of these constructs. The spacing of mutated sequences relative to the start of transcription was not changed. The mutant promoter plasmids were introduced into maize suspension cells with either B and C1 plasmids, or with the P plasmid, or without transacting factor plasmids. Figure 4 shows the activities of the mutant constructs from -123 to -79, relative to the unaltered 1.4-kb promoter in A1-Luc. The activities of some linker-scanner mutations were also measured in embryos and aleurones (Figure 4).

In BMS cells, the linker-scanner mutations with changes spanning -88/79, -78/69 (data not shown), and -68/59 (data not shown) resulted in promoter activities between 98 and 117%. This indicates that the -88 to -59 region contains no essential sequences for A7 promoter activity. However, any of the four site-directed mutations in the -128/-79 region reduced

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both B/C1- and P-mediated transactivation to some extent. The overall expression from the promoter constructs mutated in this region was never higher than 60% for B/C1-mediated transactivation and 32% for P-mediated transactivation. The changes in the -128/119 and -118/109 region reduced P transactivation most severely to 15 and 12% of normal. The mutations in the -98/89 region reduced B/C1 transactivation most severely to 3 to 8% of normal (Figure 4). These results, in addition to the 5' deletion at -123, indicate that the sequences between -123 and -109 are most important for P transactivation, whereas sequences between -98 and -89 are most important for B/C1 transactivation.

Four of the linker-scanner mutations were also assayed in aleurones and embryos expressing the R and C1 genes (Figure 4). A self-colored allele of R (R<sup>com2</sup>) is needed to obtain anthocyanin expression in the embryo because the standard R allele is not expressed in embryos. This experiment measures the effect of the promoter mutations in a cell expressing the R and C1 proteins from the endogenous genes at normal levels. The embryo and aleurone results gave lower but generally similar ratios of mutant to wild-type promoter expression as was observed in BMS cells (Figure 4). One major difference was that the -118/109 site was more important relative to the -108/99 site in aleurones and embryos than in BMS cells for B/C1 transactivation. These results generally confirm the importance and magnitude of these sequence changes on promoter transactivation in the presence of endogenous levels of R and C1 proteins.

Oligonucleotide Trimer Fusions to the CaMV 35S Core Promoter

To further confirm regions of the A1 promoter that are sufficient to mediate transactivation by B/C1 and P, small oligonucleotides were tested for their ability to induce a minimal CaMV 35S promoter. These oligonucleotide trimers were tested as trimers upstream of the minimal CaMV 35S construct (−73 5' end) and were aligned by the homology area shown by the vertical lines. Each oligonucleotide trimer clone was bombarded at least five times into BMS-EG5 cell suspensions, with or without transactivator genes. All values are shown as the fold induction of LUC activity relative to bombardment without the regulatory gene alone led to only a two- to fivefold enhancement of LUC activity relative to bombardments without the activator plasmids (data not shown). These oligonucleotide trimer experiments demonstrated that the −140 to −79 region of the A1 promoter appears to contain the most important regions needed for B/C1 and P transactivation. The sequences most important for P-mediated activation are in the −100 to −134 region, with increased stimulation when the −79 to −99 region was included. The −134 to −100 region conferred some B/C1 inducibility, but the inducibility was 12-fold stronger when the −99 to −88 region was included. The ability of both A1 trimers to stimulate expression to different levels suggests the presence of more than a single area of contact of the regulatory proteins in this region of the promoter.

**DISCUSSION**

Mutational analysis of the A1 promoter has established that the −123 to −88 region is critical for the activation of the A1 gene by the anthocyanin and phlobaphene regulatory genes. An oligonucleotide trimer from −140 to −79 confers efficient B/C1- or P-dependent activation to a minimal CaMV 35S promoter. The location of this critical region between −123 and −88 is consistent with the varying levels of anthocyanin expression in the embryo because the standard R allele is not expressed in embryos. This experiment measures the effect of the promoter mutations in a cell expressing the R and C1 proteins from the endogenous genes at normal levels. The embryo and aleurone results gave lower but generally similar ratios of mutant to wild-type promoter expression as was observed in BMS cells (Figure 4). One major difference was that the −118/109 site was more important relative to the −108/99 site in aleurones and embryos than in BMS cells for B/C1 transactivation. These results generally confirm the importance and magnitude of these sequence changes on promoter transactivation in the presence of endogenous levels of R and C1 proteins.

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biosynthesis observed in revertants of the a1-m2 mutant allele carrying a defective Suppressor-mutator (dSpm) element at −99 (Masson et al., 1987). Presumably, the imprecise excisions of the dSpm element account for the reduced and variable expression observed in the revertants. The A1 promoter does not appear to be negatively regulated because in the absence of B/C1 or P all of the mutant promoters are expressed at the same level as the intact A1 promoter, which is 50-fold less than the induced levels.

The −123 to −88 region of the A1 promoter contains two myb-like consensus DNA binding sites but does not contain bHLH-like consensus DNA binding sites. Previously, the presence of myb-like and bHLH-like consensus DNA binding sites in the critical region of the Bz1 promoter was consistent with a model of B and C1 interactions with the promoter based on their homologies to the DNA binding regions of the bHLH and MYB proteins. This model is still likely for the A1 promoter's interactions with C1, P, or B at the myb-like consensus sites spanned by the site-directed mutations at −118 to −109 and −108 to −99 and the P binding site at −65 to −55 (Grotewold et al., 1994). Mutations at any of these sites, particularly the upstream sites, affect both P and B/C1 induction of the A1 promoter.

The P protein was observed to bind in vitro only to the −55 to −65 region and not to the region between −123 to −88 (Grotewold et al., 1994). However, a site-directed A1 promoter mutation that abolished P binding in vitro still retained 40 and 75% of wild-type A1 promoter activity in the presence of P or R/C1, respectively (Grotewold et al., 1994). An indication that the −55 to −65 site is not sufficient for A1 promoter induction is that five deletion mutants lose 90% of activity at nucleotide −98 and virtually all activity at nucleotide −78. The −55 to −65 site is not required because the −88 3' A1 promoter deletion–Adh1 core promoter construct removes this region and retains 70% of the P- or B/C1-dependent enhancer function. Similarly, the region between −65 and −55 is not present in the inducible oligonucleotide trimer constructs (this study) but does confer some P or B/C1 inducibility as an oligonucleotide trimer (Grotewold et al., 1994). Thus, whereas P binds to the region between −55 and −65 in vivo, it is clear that it must also act through the more important −123 to −88 region in vivo. The lack of P binding to the −123 to −88 region in vitro may be a result of the absence of other required factors or protein modifications not present in the P protein produced in E. coli. It is worth noting that a P-like site (Grotewold et al., 1994) of the sequence AACTACC at nucleotides −123 to −116 overlaps with the myb-like site that we have identified at nucleotides −119 to −115 (Figure 1A).

The critical −123 to −88 region of the A1 promoter does not contain a bHLH-like consensus DNA binding site (CANNTG). This result is consistent with the recent observation that the bHLH region of the B protein is not essential for B transactivation of anthocyanin structural gene promoters. A deleted B protein missing the bHLH domain was still able to transactivate the Bz1 promoter to 50% (Goff et al., 1992) and the A1 promoter to 70% (J.A. Tuerck, unpublished results).

of the level observed with intact B protein. Thus, the original proposal, that the B/R proteins bind to bHLH-like consensus sites in anthocyanin promoters through their bHLH domain, is not valid. This is consistent with the lack of well-defined bHLH-like consensus DNA binding sites in the other anthocyanin promoters. However, these bHLH-like sites and domains may play a role in some of the fine tuning of the individual genes like Bz1 under some of the diverse light, stress, and tissue-specific regulation that occurs in anthocyanin production (Taylor and Briggs, 1990).

Although the bHLH-like consensus DNA binding site is not conserved, alignment of this region of the Bz1 promoter and the critical region of the A1 promoter reveals an 11-bp of 15-bp match (Figure 6). This area (−100 to −86) in the A1 promoter produced the most severe reduction in A1 expression when changed by a linker-scanning mutation (3 to 8% residual activity with B/C1). Linker-scanner mutations in the Bz1 homologous region (−64 to −50) reduced expression to 1 to 3% of normal (Roth et al., 1991). Thus, the sequences most important for B/C1 inducibility show a significant homology in the two promoters. However, the nonidentical nucleotides create a bHLH-like consensus binding site in the Bz1 promoter that is not present in the A1 promoter (Figure 6). The severe effect of the linker-scanning mutation in the 10-bp region of the Bz1 promoter that contains the bHLH consensus binding site (−53 to −58; Roth et al., 1991) is now attributed to the mutations in the nucleotides conserved between the A1 and Bz1 promoters and not to the concurrent changes in the bHLH consensus binding site.

Inspection of the A2, Bz2, and C2 promoters revealed that the sequences required for B/C1 inducibility of the A1 and Bz1 promoters have homology with sequences in these promoters (Figure 6). The five anthocyanin promoters match a core consensus sequence (Figure 6). The consensus sequence was derived from the regions of the five anthocyanin promoters shown. The regions were identified by homology to the aligned regions (vertical lines) of the A1 and Bz1 promoters that were found to be critical for B/C1 inducibility in vivo. The nucleotide numbers are relative to the start of transcription. Nucleotides matching the consensus sequence are represented as uppercase letters. The myb-like and bHLH-like consensus DNA binding sequences are shown above the Bz1 oligonucleotide. Potential myb-like sequences in the A1 oligonucleotide are underlined and are on the opposite strand relative to the myb-like site in the Bz1 sequence.

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**Figure 6. Consensus Sequence for Anthocyanin Promoters.**

The consensus sequence was derived from the regions of the five anthocyanin promoters shown. The regions were identified by homology to the aligned regions (vertical lines) of the A1 and Bz1 promoters that were found to be critical for B/C1 inducibility in vivo. The nucleotide numbers are relative to the start of transcription. Nucleotides matching the consensus sequence are represented as uppercase letters. The myb-like and bHLH-like consensus DNA binding sequences are shown above the Bz1 oligonucleotide. Potential myb-like sequences in the A1 oligonucleotide are underlined and are on the opposite strand relative to the myb-like site in the Bz1 sequence.
consensus sequence (CGACTGCCNGGTGC) in at least nine of the 14 nucleotides. Four of the positions in the consensus sequence are conserved, and four more match four of the five promoter sequences. The consensus sequence is located within 100 bp of the start of transcription in four of the five promoters, with the exception of the C2 promoter, in which the consensus sequence starts at -263. In addition, the A1, BZ1, BZ2, and A2 promoters have a myb-like consensus sequence located within 32 nucleotides upstream of the location of their 14-bp consensus sequence (at -98 in BZ2 and at -105 in A2).

The question of how the anthocyanin and phlobaphene transcription factors interact with the structural gene promoters remains. There are limited data on the in vitro binding of B and C1 (Wienand et al., 1990). P binding has not been observed in vitro at the -123 to -88 region (Grotewold et al., 1994), although our results indicate that this region is essential for A1 promoter activity in vivo. However, some properties of these regulatory proteins are known from protein domain substitution studies. The C1 acidic region forms a transactivating factor that functions independently of B when attached to the GAL4 DNA binding domain (Goff et al., 1991) or the P DNA binding domain (Grotewold et al., 1994). However, the C1 DNA binding domain, which is 80% identical to that of the P protein, still required the B protein for transactivation of the BZ1 promoter when attached to the GAL4 acidic region (Goff et al., 1991). The dependence of C1 on B is likely to be mediated by a direct interaction because C1 and B have been shown to interact in vivo in protein tethering experiments (Goff et al., 1992). In addition, the B protein did not contain any transactivating regions when fused to the GAL4 DNA binding domain (Goff et al., 1992).

A possible model based on the above promoter and protein domain data is that C1 contains a functional acidic transactivating region but binds myb-like consensus sites inefficiently. The B protein contains no transactivating domain but can bind to C1 and to DNA at the 14-bp consensus sequence (CGACTGCCNGGTGC), with a DNA binding domain other than the nonessential bHLH domain. (If B does not bind at the 14-bp consensus site, then a constitutive factor must be binding at the consensus site.) The B/C1 complex binds to the promoter DNA with higher affinity and specificity than C1 alone at myb-like sites and optimally with B contacting the anthocyanin 14-bp consensus site. In addition, the oligonucleotide trimer results indicate that B/C1 complex can bind DNA with lower efficiency at myb-like sites in the absence of the 14-bp consensus sequence. The dependence of C1 on B allows C1 to recognize myb-like sites that are different from the ones to which P binds.

In this model, the P protein, which is 80% identical to C1 in the MYB-homologous N-terminal region, has a higher affinity and specificity than C1 for binding myb-like sites (or the overlapping P-like consensus sites at -60 and -119; Figure 1A). Thus, the P protein can bind and transactivate promoters in the phlobaphene pathway without interaction with a B protein. The greater specificity of the P protein also allows it to distinguish between phlobaphene myb-like binding sites, presumably P-like sites such as those in the A1 promoter, and those unique to the anthocyanin pathway, such as those in the BZ1 promoter. It is of course possible that both B/C1 and P depend on a yet to be identified factor, which interacts with the conserved 14-bp motif (CGACTGCCNGGTGC), for productive binding and transactivation events. However, this factor must be constitutive because most plant cells produce anthocyanin when B and C1 are introduced into the cells transiently. Similarly, the transactivation of the A1 promoter in BMS cells requires only the addition of B/C1 or P.

Further studies of these promoters and other anthocyanin and phlobaphene structural gene promoters such as Bronze-2 (Bodeau and Walbot, 1992) and their binding proteins will be needed to fully resolve these complex interactions. This study defines the critical regions necessary for A1 promoter activity in vivo, which should help develop accurate in vitro binding systems. These results also provide a basis for determining consensus sites that the B/C1 and P proteins act through to regulate the various structural genes in the anthocyanin and phlobaphene biosynthetic pathways.

METHODS

Plasmids

Plasmids used in this study that have been previously described are as follows: p355-C1 and p35S-B-Peru, (Goff et al., 1990); pAdh-CAT, (previously described as pAV1CN, Callis et al., 1987); p35S-P cdNA1 (Grotewold et al., 1991); pBZ1-Luc; (Klein et al., 1988). A1-Luc (Klein et al., 1989) was modified to contain NcoI, SacII, and HindIII sites 5' of the A1 promoter. pSG2-2 contains NcoI, SacII, AvaI, NheI, SpeI, Xhol, and HindIII sites upstream of the cauliflower mosaic virus (CaMV) 355 minimal promoter (Roth et al., 1991). pSG2-7 contains NcoI, SacII, AvaI, NheI, SpeI, Xhol, and HindIII sites upstream of the -89 alcohol dehydrogenase 1 (Adh1) core promoter (Roth et al., 1991).

5' Deletions of the 1.4-kb A1 (Klein et al., 1989) promoter fragment were produced by ligating Xhol linkers to blunt-ended Bal31 exonuclease digestion products of A1-Luc DNA. 3' Deletion products were produced by the polymerase chain reaction with an Xhol site at the deletion boundary. Site-directed mutagenesis (Kunkel et al., 1987) of the A1 promoter replaced 10 nucleotides of the A1 promoter with an Xhol site (CCCTCGAGGGG) in each mutant. Two oligonucleotide sequences, a 67-mer oligonucleotide (GATCTCGCCCGCAAGATATGCGGTAGTTGCAAGCCTGTGTTTGTTAATGGAAGATGCCGCTACCG), the A1 promoter sequences from -146 to -79 and a 41-mer (GATCCTCAAGATATGCCCGGTAGTGCAAGCCTGTGTTTGTTAATGGAAGATGCCGCTACCG) from -134 to -100, both flanked by BgII and BamHI sites, were trimerized in direct repeats and cloned into the BamHI site of pCi19H+ (Marsh et al., 1984). The Xhol- and Sall-digested trimer DNA was ligated upsteam of the -73 CaMV 355 core promoter of pSG2-2, with the sequences in their normal orientation.

Plant Material and Cell Bombardment

The Black Mexican Sweet maize suspension line (BMS-EG5, Chourey and Zurawski, 1981) used was able to produce anthocyanin pigments
after bombardment with BIC1 expression plasmids. For the biolistic bombardment of embryogenic callus or BMS suspension cells, 3 μg of the experimental plasmid, 3 μg of either p35S-C1 and p35S-B-Peru or p35S-P, and 2 μg of pAdh-CAT DNA were precipitated and delivered to the cells as previously described (Klein et al., 1989; Goff et al., 1991). For bombardment of bz7C1, R°embryos (Roth et al., 1991) or atC1, R aleurones (Klein et al., 1989), 6 μg of the experimental plasmid and 2 μg of pAdh-CAT were coprecipitated onto tungsten particles. Each construct was bombarded in at least five independent bombardments into BMS-EG5 cells, or in at least three independent experiments into embryos or aleurones. One independent experiment comprised three replica bombardments. The mean of those experiments and the standard error of the mean were calculated and are presented in the figures.

Enzyme Assays

LUC was assayed in cell extracts (Callis et al., 1987), and its activity was expressed as the number of light units detected in 10 sec at 25°C. Chloramphenicol acetyltransferase (CAT) activity was determined by the amount of counts per minute of 14C-labeled acetyl coenzyme A converted to 14C-acetoxy-chloramphenicol in 1 hr at 37°C (Sleigh, 1986). The LUC-to-CAT ratio was calculated as previously described (Roth et al., 1991).

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