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A GBF-Binding Site and a Novel AT Element Define the Minimal Sequences Sufficient To Direct Prespore-Specific Expression in *Dictyostelium discoideum*

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In order to better understand the molecular mechanisms of cellular differentiation in *Dictyostelium discoideum*, we have identified the minimum regulatory sequences of the prespore-specific gene *SP60/cotC* that are sufficient to confer cell-type-specific expression on a heterologous promoter. This region includes at least two essential *cis*-acting elements: a novel AT-rich element (or elements) and CAE3. The essential function of the AT element is confirmed through point mutations that decrease expression below the level of detection. CAE3 is one of three CA-rich elements (CAEs) required for the induction of *SP60/cotC* during development or in response to extracellular cyclic AMP. The CAEs have differential affinities for a specific developmentally induced nuclear activity (CAE1 > CAE2 >> CAE3). Here, we identify this activity as G-box-binding factor (GBF) and show that in vitro-transcribed and -translated GBF binds all three *SP60/cotC* CAEs in a sequence-specific manner. Previous studies have suggested that GBF mediates the induction of some prestalk genes, and these results demonstrate that it also has a specific role in prespore gene activation.

Successful multicellular development requires the organization and patterning of many divergent cell types. Individual cells must respond to inductive signals and initiate cascades of intracellular events that ultimately influence programs of gene activation and cellular differentiation. The cellular slime mold *Dictyostelium discoideum* has proven to be a useful system for studying this complex process. *D. discoideum* cells undergo a relatively simple program of multicellular development and are amenable to molecular and biochemical manipulation. However, the transcription factors that mediate differentiation in *D. discoideum* have not yet been identified, and this has limited progress towards understanding the molecular mechanisms of cell type specification.

In the presence of nutrients, *D. discoideum* cells exist as individual amoebae, but when starved, up to 10^5 cells aggregate to create a multicellular organism. Each cell must differentiate into one of a few cell types. Approximately 20% of the cells differentiate into one of three subtypes of prestalk cells or anterior-like cells (9, 28). The remaining 80% of the cells assume the prespore cell fate and are localized to the posterior of the organism (12, 14, 15, 26, 34, 42). Intrinsic factors help to establish the appropriate initial proportions of the cell types: a cell that is starved late in the G_2 stage of the cell cycle is predisposed to a prespore cell fate, while cells starved early in the cell cycle have a propensity to differentiate into prestalk cells (13, 41). Extracellular signals also influence differentiation. Prespore gene expression is induced by high levels of extracellular cyclic AMP (cAMP) (4, 11, 15, 32, 33, 38) and is inhibited by the chlorinated alkyl phenone differentiation-inducing factor (8, 11). Some of the intracellular signal transduction molecules essential for prespore/spore differentiation have also been identified (5, 17, 21, 29–31).

Six prespore genes have been cloned, and preliminary deletion analyses have localized the essential *cis*-elements to <690 bp in each case (10, 12, 15, 34, 37, 40). However, the promoter elements sufficient to direct prespore-specific transcription have not yet been defined. The only *cis*-elements that have been functionally identified thus far with point mutations are the CA-rich elements (CAEs) in the *SP60/cotC* promoter (15, 16). The three CAEs in the *SP60/cotC* promoter share an extensive consensus sequence (two CACACA repeats separated by 5 to 6 nucleotides [see Fig. 1 and reference 15]), and small deletions or point mutations in any one of them result in a 10- to 25-fold decrease in promoter induction during development or in response to high levels of cAMP. Although similar in these ways, the *SP60/cotC* CAEs are functionally distinct. Point mutations in the distal-most CAE (CAE1) unmask an anterior-to-posterior gradient of expression in the prespore zone, mutation of CAE2 decreases transcription levels 10-fold without changing the expression pattern of the promoter, and a small deletion of CAE3 results in a subtle posterior-to-anterior gradient.

The three CAEs were also shown to have different affinities for nuclear activities in gel shift assays. CAE1 and CAE2 have been shown to bind a nuclear activity that is induced during development or in response to extracellular cAMP. Moreover, a correlation was demonstrated between the nucleotides within the CAEs that are important for in vivo activity and the nucleotides required for interaction with this CAE-binding activity in vitro, indicating that this nuclear activity might mediate developmental activation of the *SP60/cotC* promoter through CAE elements. CAE3 has a relatively weak affinity for the developmentally induced activity, and it has been proposed to have a regulatory function distinct from that of CAE1 and CAE2 (15, 16).

We have further analyzed the *SP60/cotC* promoter to identify and characterize the *cis*-acting elements minimally sufficient to direct prespore-specific expression. Previous studies

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have indicated that CAE1 and CAE2 are discrete activating elements, as large deletions that included CAE1 or CAE2 did not decrease promoter activity substantially more than point mutations. We show here that the region around CAE3 is more complex than CAE1 or CAE2 and that multiple elements act synergistically to activate transcription. Furthermore, we delimit the regulatory sequences sufficient to confer cell-type-specific expression to a heterologous promoter, and we demonstrate that both CAE3 and a novel 37-bp AT-rich regulatory region are required for this function. In addition, we show that point mutations within the AT element prevent gene expression.

We identify the developmentally induced CAE-binding activity as G-box-binding factor (GBF) and show that it binds the three *SP60/cotC* CAEs with sequence specificity. GBF had been described previously as a developmentally regulated, cAMP-induced activity that interacts with *cis*-elements required for the developmental induction of the prestalk-enriched genes *CP2/pst-cathepsin* and *ecmB* (3, 20). Since one GBF binding site from *DG17* (20) fits the *SP60/cotC* CAE consensus well, and since GBF has a competition profile similar to that of the CAE-binding activity (see Results), we hypothesized that the CAE-binding activity and GBF were identical. GBF has recently been purified, and the gene encoding it has been cloned (39). Here, we demonstrate that GBF copurifies with the CAE-binding activity and that recombinant GBF binds the *SP60/cotC* CAEs with properties similar to those of the activity observed in crude nuclear extracts.

MATERIALS AND METHODS

Construction of plasmids. The construction of 5′-deleted *SP60/cotC* expression vectors was described previously (15). ExoIII nuclease was used to generate additional 3′ deletions from the *Spe*I site in the 5′Δ20 promoter, and the deletions were capped with *Hin*dIII linkers. The NdH endpoint was made by filling in the *Nde*I site with the Klenow fragment of DNA polymerase I and adding a *Hin*dIII linker. To create internally deleted promoters, 3′ deletions were cloned into the *Bam*HI and *Hin*dIII sites upstream of the 5′ deletion endpoints in expression vectors. (See Fig. 1 for deletion endpoints.)

3′ deletions of 5′Δ20 were cloned into A15ΔBam-gal (2) as follows. The 3′-deleted promoters were subcloned into the *Bam*HI and *Spe*I sites of Bluescript (Stratagene). The resultant vectors were cut with *Spe*I, blunt ended with the Klenow fragment, and capped with *Bgl*II linkers. The *Bam*HI-*Bgl*II fragments were cloned into the *Bam*HI site of A15ΔBam-gal, to create the Act15-60Δ_ constructs. To construct Act15-60-Δ13Δ21 and Act15-60-3(Δ13Δ21), the 3′Δ13 *Bam*HI-*Hin*dIII fragment was cloned into Bluescript. The *Hin*dIII site was filled in, and *Bgl*II linkers were added. This vector was cut with *Nde*I and *Sca*I, and the 3′Δ13-containing fragment was ligated to the *Nde*I-*Sca*I fragment of a 5′Δ21 pGEM7 subclone. The *Bam*HI-*Bgl*II fragment containing the 5′Δ21-3′Δ13 sequences was cut out and ligated into A15ΔBam-gal in one copy or three tandem copies.

Deletion fragments of 5′Δ21-3′Δ13 were generated by PCRs. 5′ deletions were amplified from pGEM7 subclones with the 3′ primer 5′-GTTTAGATCTAAAAAATAATTAT TAAATTTGTTCTATG-3′ and the SP6 primer. Internally deleted fragments were amplified from expression vectors with the 3′ primer and a 5′ primer, 5′-GTTTGGATCCATATATA TACACTGTGAG-3′. The PCR products were subcloned (*Bam*HI and *Bgl*II), sequenced, and cloned as three tandem repeats into A15ΔBam-gal. To create the ΔNdH-42(b3) mutant, the Δ42-Δ110 sequences were removed from the ΔNdH-42 subclone (*Hin*dIII and *Bgl*II) and replaced with the mutant oligonucleotide (mutated nucleotides are underlined and in boldface) agctTTTTGTAAAA**A**ATAGAA**A**AAATT **G**AATAA**G**TATTTTTTAgatc.

The pSP72N2B vector was designed to allow the conversion of a *Hin*dIII site to a *Bam*HI site with minimal additional nucleotides. The *Hin*dIII site of pSP72 (Promega) was filled in with the Klenow fragment of DNA polymerase I, and a *Hin*dIII linker was cloned into the blunt-ended *Bam*HI site, recreating *Bam*HI sites on either side of the *Hin*dIII site. The CAE3-containing 3wt oligonucleotide (16) was cloned into pSP72N2B, cut out as a *Bam*HI fragment, and cloned into A15ΔBam-gal.

β-Galactosidase staining. Cells were plated for development on white Millipore filters placed on phosphate-buffered agar. At the appropriate times, the organisms were stained for β-galactosidase activity by a modification of previously described procedures (7, 15). Filters were removed from the agar and allowed to air dry for 1 min and then placed on Whatman filters saturated with 0.5% glutaraldehyde–0.05% Triton X-100 in Z buffer. After 5 min, additional fixation solution was added to the filters to immerse the organisms for 10 min. The cells were washed in Z buffer and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution (1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, and 0.5 mg X-Gal per ml in Z buffer) for up to 3 h at 37°C.

Luciferase activity measurement. Luciferase activity was determined as previously described (15, 22). All relative expression data are mean values from at least two independently derived populations of stable transformants, each assayed in at least two separate experiments. All values were normalized for the copy number of the vector, as determined by Southern blot hybridization. Copy numbers never varied by more than fivefold, compared with the control construct, and usually varied by less than twofold.

Nuclear extract preparation. Nuclear extracts were prepared as described previously (19), except that Tris buffer was used instead of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and extracts were not heat treated. Extracts were diluted to 2.5 mg/ml in buffer I50 for precipitation at increasing concentrations of ammonium sulfate. Fractionation of extracts to enrich for GBF activity is described elsewhere (39).

In vitro transcribed-translated GBF. The vector R20 containing the complete coding region of GBF (39) was linearized with *Apa*I. In vitro transcription and translation were then performed with the TNT T3-coupled reticulocyte lysate system (Promega). For binding to CAE3 in vitro, transcription and translation reaction mixtures with R20 or a luciferase control vector were diluted 10-fold in 120 mM KCl affinity column buffer (ACB) (5% glycerol, 3.3 mM Tris [pH 8.4], 6.7 mM Tris [pH 7.5], 0.1 mM EDTA, 1 mM dithiothreitol, and 0.05% Triton X-100) and applied to a CP2 G-box affinity column equilibrated in the same buffer. The column was washed with 10 column volumes of 120 mM KCl ACB supplemented with 1 mg of bovine serum albumin (BSA) per ml and eluted with 2 column volumes of 420 mM KCl ACB with 1 mg of BSA per ml. The affinity column was prepared with the complementary CP2 G-box oligonucleotides 5′ GATCCACAGCGGGTGT GTTAAGTTAGGGGTGGGTTTTATATAGGT and 5′ GA TCTCCTATATAAAACCCACCCCTAACTTAACACACC CGCTGTG according to previously described methods (39).

Gel retardation assays. Gel mobility shift assays were performed as previously described (16) except as detailed in the figure legends. The shorter CAE3-containing oligonucleotide described in the text is agctACAAACACACTCCCAACACA

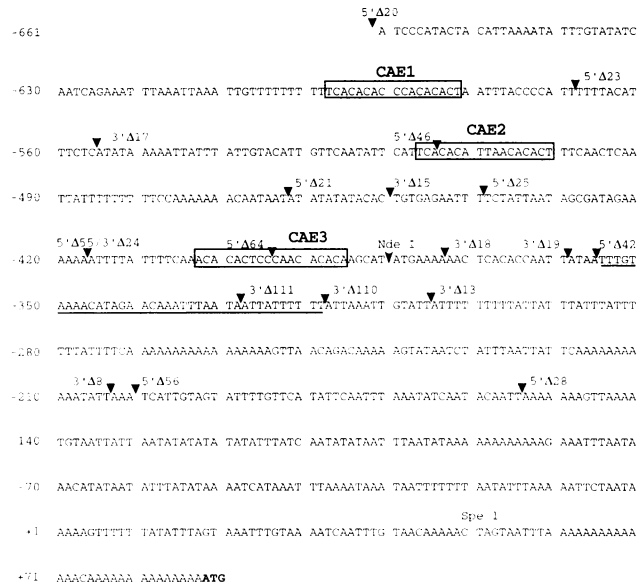

FIG. 1. Deletion endpoints in the *SP60/cotC* promoter. The three CAEs are boxed, and the AT element is underlined. The transcriptional start site is labelled +1. The *Nde*I endpoint is also shown (see Materials and Methods).

CAAGCAtgca (5′ overhangs are lowercase). The other *SP60/cotC* CAE-containing oligonucleotides (16) and the *CP2* G-box-containing oligonucleotides (19) have been described previously.

RESULTS

Localization of *SP60/cotC* promoter elements. Previous analyses have shown that 661 bp of *SP60/cotC* 5′ flanking sequence is sufficient to direct high levels of cell-type-specific expression and that the three CAEs are required for full developmental induction. It was also shown that the CAEs, while necessary for detectable levels of expression, were not sufficient to direct prespore-specific transcription: a large deletion 3′ of the CAEs caused a catastrophic ($>10^4$-fold) reduction in expression (15, 16).

In order to localize those regions of the *SP60/cotC* promoter required for prespore-specific transcription, we assayed a series of internal deletions with *luciferase* and *lacZ* reporter constructs. Since it was previously shown that the sequences downstream of 5′Δ21 were sufficient to direct prespore expression, although at diminished levels (15), we focused our studies on this region of the promoter. The deletions and the resultant relative expression levels are listed in Fig. 1 and 2. Removal of all or part of CAE3 reduced promoter activity 15- to 40-fold (compare full-length with Δ24-64 and Δ15-64 or compare Δ15-55 with Δ15-NdH). The region immediately upstream of

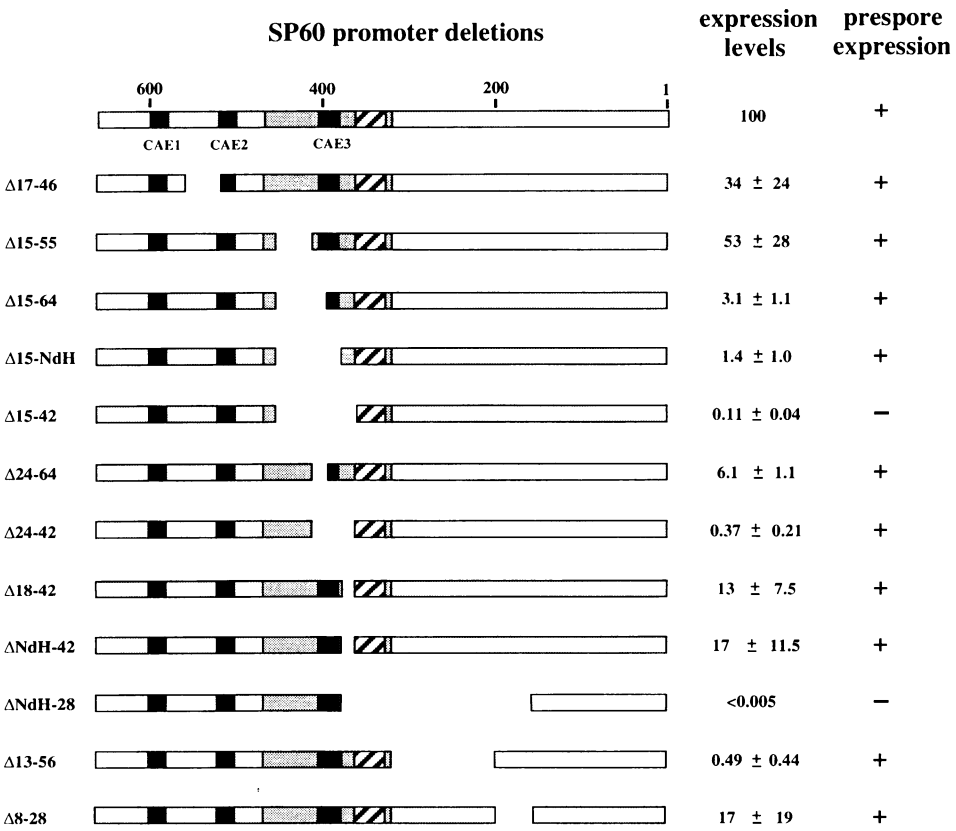

FIG. 2. Relative levels of developmentally induced expression directed by internally deleted *SP60/cotC* promoters. The three CAEs are depicted as black boxes. The region between the 5′Δ21 and 3′Δ13 endpoints is shaded gray, and the position of the AT element is indicated with a striped box. Relative expression values and standard errors, as assayed by developmentally induced *SP60/cotC*-luciferase levels, are listed. + indicates that the expression, as detected by *SP60/cotC*-β-galactosidase staining, was prespore specific. Constructs that did not express at high enough levels to determine cell type specificity are labelled −.

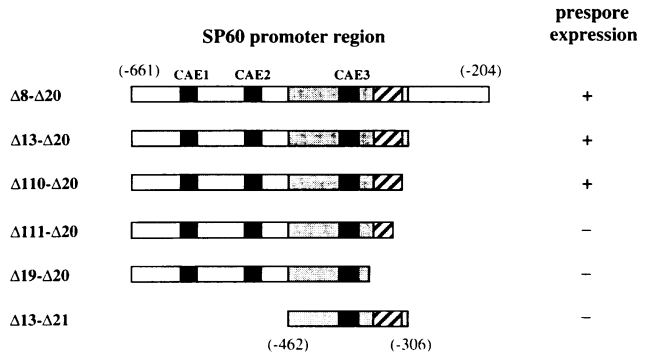

FIG. 3. Ability of *SP60/cotC* promoter regions, in one copy, to confer prespore-specific expression on the *Actin15* minimal promoter. See legend to Fig. 2 for schematic representation of promoter elements. The 5′ and 3′ deletion endpoints that define each fragment are indicated, with the 3′ endpoint listed first. + indicates that one copy of the fragment conferred prespore-specific expression on the heterologous promoter, and − indicates that one copy of the fragment did not confer detectable levels of developmentally induced expression.

CAE3 (Δ15-55) could be deleted with only a two- to threefold decrease in promoter activity, and removal of the 25 bp immediately downstream of CAE3 (ΔNdH-42) reduced expression ~6- to 10-fold. Larger deletions revealed that these decreases were multiplicative (the promoter activity of Δ15-42 is 0.11% of that of the full-length promoter). In all cases, *SP60/cotC/lacZ* expression, when detectable by X-Gal staining, was prespore specific (Fig. 2).

As previously reported (15), a large deletion of the 226 bp downstream of CAE3 (ΔNdH-28) decreased promoter activity to below detectable levels (<0.005%). Smaller deletions within this region indicated that multiple elements downstream of CAE3 were required for full promoter activity.

Determination of minimal sequences sufficient to direct prespore expression. Both the Δ13-56 and the Δ8-28 internally deleted promoters directed prespore-specific expression (Fig. 2), suggesting that the sequences upstream of 3′Δ13 might be sufficient to direct cell-type-specific expression. To test this, we used the *Actin15* minimal promoter construct, A15ΔBam-gal (2) (also termed Actin15ΔBgal [37]), to assay the ability of *SP60/cotC cis*-acting regions to confer developmentally regulated, cell-type-specific expression. The A15ΔBam-gal promoter construct does not express any intrinsic, detectable β-Gal activity. Moreover, since the *Actin15* promoter is cell type nonspecific in its expression pattern, A15ΔBam-gal is a suitable minimal promoter for assaying cell-type-specific regulatory elements from heterologous promoters (2, 10). The sequences between 5′Δ20 and 3′Δ13 were cloned into A15ΔBam-gal and assayed for β-galactosidase expression in whole-mount preparations (see Materials and Methods). This promoter region directed high levels of prespore-specific expression (Fig. 3; data not shown). We then assayed additional 3′ deletions to determine the 3′ endpoint of the prespore regulatory sequences. The region between 5′Δ20 and 3′Δ110, which includes all three CAEs and 67 bp of sequence downstream of CAE3, conferred high levels of cell-type-specific expression on the minimal promoter. However, a 10-bp deletion to 3′Δ111 reduced expression to below detectable levels (Fig. 3; deletion endpoints in Fig. 1).

Previous studies had shown that the sequences downstream of 5′Δ21 were sufficient to direct cell-type-specific expression, although expression levels were 500-fold less than those conferred by the full promoter (15). To determine whether the region between 5′Δ21 and 3′Δ13 included all the information sufficient for prespore-specific expression, we cloned this fragment into the minimal promoter to create Act15-60-(Δ13-Δ21). The internal deletion data listed in Fig. 2, taken with previous 5′ deletion studies (15), suggested that the sequences between 3′Δ13 and 5′Δ21 might not provide enough enhancer activity to yield detectable β-galactosidase staining. Indeed, transformants carrying this construct did not express detectable levels of β-galactosidase (Fig. 3; data not shown). To increase the levels of transcriptional activation provided by this region, we cloned three tandem repeats into the A15ΔBam-gal minimal promoter. The resultant construct directed high levels of prespore-specific expression (Fig. 4A; data not shown; see below).

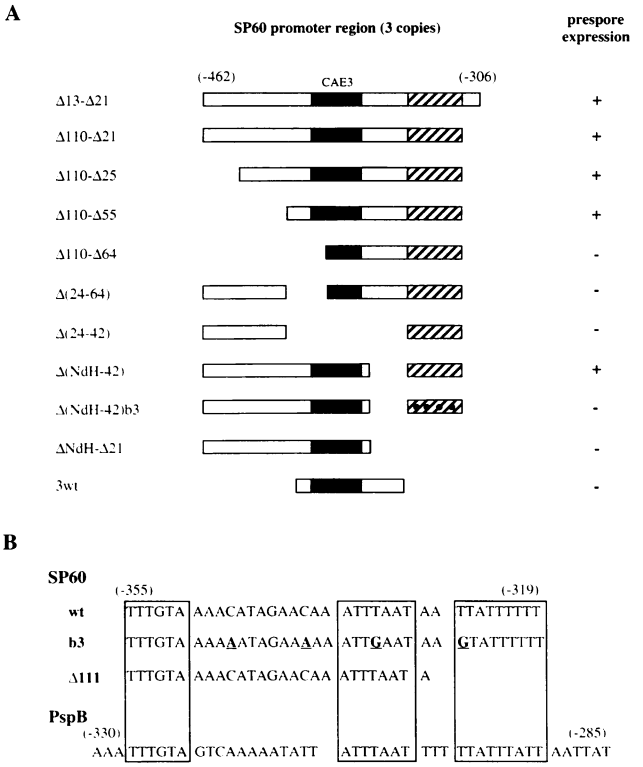

FIG. 4. (A) Ability of *SP60/cotC* promoter regions, in three tandem repeats, to confer prespore-specific expression on the *Actin15* minimal promoter. The 5′ and 3′ deletion endpoints that define each promoter fragment are indicated, with the 3′ endpoint listed first. Internal deletions of the 5′Δ21→3′Δ110 fragment are also shown with the endpoints indicated in parentheses. CAE3 is schematically represented as a black box, and the AT element is represented as a striped box. The Δ(NdH-42)b3 fragment differs from Δ(NdH-42) by four point mutations in the AT element (see panel B). 3wt is a 54-bp CAE3-containing oligonucleotide (16). + indicates that three copies of the fragment conferred prespore-specific expression on the heterologous promoter, and − indicates that three copies of the fragment did not confer detectable levels of developmentally induced expression. (B) Comparison of the wild-type (wt) *SP60/cotC* AT element with the mutated b3 element (A) and the 3′Δ111 construct (Fig. 3) and with the wild-type *PspB* AT element (determined by mutational analysis in reference 37). The four mutations introduced in the b3 construct are in boldface and underlined. The boxed sequences are identical in the *SP60/cotC* and *PspB* AT elements, with the exception of a single T/A difference at position −317 bp in *SP60/cotC*.

To further delineate the minimal *SP60/cotC* promoter se-

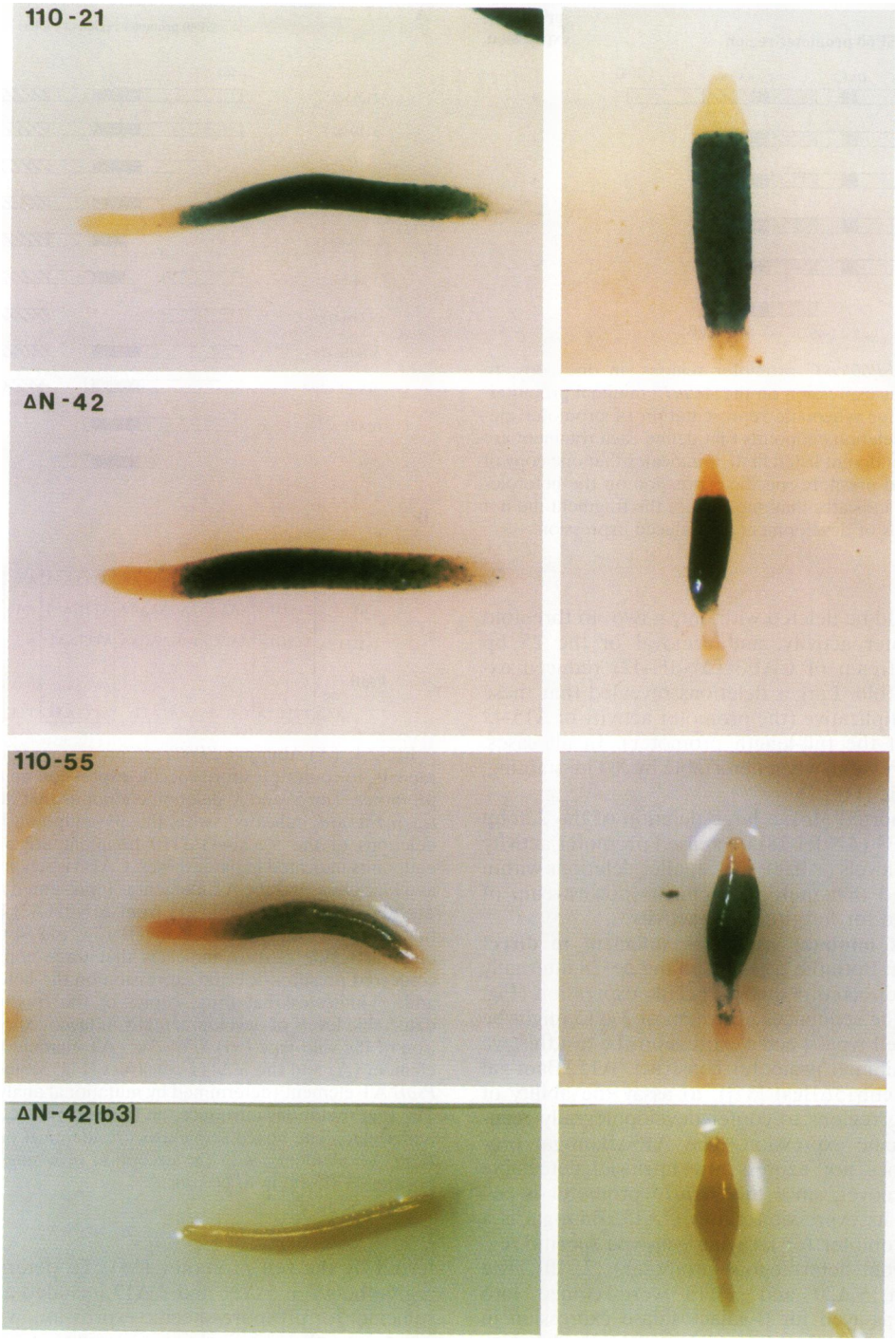

FIG. 5. Spatial pattern of expression conferred by three tandem repeats of *SP60/cotC* promoter regions at the slug and early culminant stages of development, as detected by β-galactosidase staining of transformants. In the migrating slug, the prespore cells are localized to the posterior 80% of the organism. Once the organism reaches a location suitable for culmination, it forms a second finger or early culminant before the tip cells funnel down through the prespore cells and differentiate into stalk. Both developmental stages are shown here for cells transformed with the constructs Act60-3(110-21), Act 60-3(110-55), Act60-3[Δ(NdH-42)], and Act60-3[Δ(NdH-42)b3]. For a description of the constructs, see Fig. 4A.

quences sufficient to direct prespore expression, we assayed deletions of the 157-bp Δ13-Δ21 fragment, each as three tandem repeats in the minimal promoter. The results of these studies are listed in Fig. 4A. As expected, the Δ110-Δ21 sequences conferred high levels of prespore expression (Fig. 4A; also pictured in Fig. 5). A 5′ deletion of the sequences upstream of CAE3 did not change the expression pattern, but the intensity of staining was reduced (see Δ110-Δ55 in Fig. 5). The CAE3 sequences were required for detectable expression, as seen in Δ(24-64), but the 25 bp immediately downstream of

CAE3, deleted in Δ(NdH-42), were not essential. By contrast, the AT-rich region between 5′Δ42 and 3′Δ110 was absolutely required to direct detectable levels of β-galactosidase expression [the Δ(NdH-42) sequences had prespore enhancer activity, but the ΔNdH-Δ21 sequences did not] (Fig. 4 and 5).

These deletion analyses indicated that two regions of the *SP60/cotC* promoter were required to confer prespore-specific expression on the minimal promoter: CAE3 and the downstream AT-rich sequences. As *D. discoideum* noncoding sequences are 80 to 95% A/T (25), we were concerned that the AT-rich regulatory region might serve a non-sequence-specific function, such as providing proper spacing in the context of the minimal promoter. To address this, we introduced four base changes (Fig. 4B) into the AT region of the Δ(NdH-42) fragment and assayed this mutant (termed b3) in the minimal promoter construct (Fig. 4A). As shown in Fig. 5, the four point mutations in the AT-rich region decreased expression to below detectable levels.

GBF binds *SP60/cotC* CAEs in vitro. Haberstroh et al. (16) had shown that multiple nuclear activities bound CAE3 in vitro. We further examined the specificity of these interactions in fractionated extracts. The only activity we found to bind specifically to the CAE3 core sequence formed a complex of relative mobility similar to and specificity identical to those of the developmentally induced activity previously shown to interact strongly with CAE1 and CAE2 (16) (Fig. 6A; data not shown). We found that a shorter (30-bp) CAE3-containing oligonucleotide had a slightly higher affinity for this activity than the 54-bp oligonucleotide used in previous studies (Fig. 6A). Similarities in developmental and cAMP regulation, relative mobility, and binding-site sequence prompted us to examine whether this activity was identical to GBF, which has been shown to mediate the developmental induction of some prestalk genes (3, 6, 18, 20, 35). GBF binding requires two half-sites of (T/G/A) G (G/T) G (T/G) G (T/G/A) (19, 20), and the *SP60/cotC* CAEs each consist of two CACACA half-sites separated by 4 to 7 nucleotides (16). In gel mobility shift assays, the *CP2/pst-cathepsin* G box (the original GBF binding site) competed strongly for binding to the CAE-binding activity, while a mutant G box (which is nonfunctional for CP2 activation in vivo and GBF binding in vitro) did not (Fig. 6A). Similarly, when the *CP2* G box was used as a probe, the CAEs competed for GBF binding (39) (data not shown). The binding affinities were as follows: CAE1 ≅ CP2 > CAE 2 > CAE 3 > CP2mut > CAE1mut.

During the course of these studies, GBF was purified and the gene encoding it was cloned (39). The developmentally regulated CAE-binding activity copurified with GBF at all tested stages (Fig. 6B; Fig. 7; data not shown). To determine directly whether the CAE-binding activity was GBF, we transcribed and translated cloned GBF in reticulocyte lysates and incubated the reaction mixtures with labelled CAE3. The lysates were enriched for GBF protein by CP2 G-box affinity chromatography (see Materials and Methods). As shown in Fig. 7A, recombinant GBF formed a complex with CAE3 that migrated at a position similar to that of the complex formed in GBF extract preparations made from developing *D. discoideum* cells. Other proteins in the reticulocyte lysate bound the probe (see control lanes in Fig. 7A), but formation of the GBF complex was dependent upon the addition of the GBF vector DNA to the reaction. Furthermore, the in vitro-transcribed and -translated GBF had a competition profile similar to that of the sequence-specific CAE3-binding activity in nuclear extracts: CAE1 and the CP2 G box had a greater affinity for GBF than CAE3, and the mutant CP2 G box competed very poorly for binding. GBF, transcribed and translated in reticulocyte lysates, also bound specifically to CAE1 and CAE 2 probes (Fig. 7B and C), confirming that the developmentally regulated activity that binds the three *SP60/cotC* CAEs is GBF.

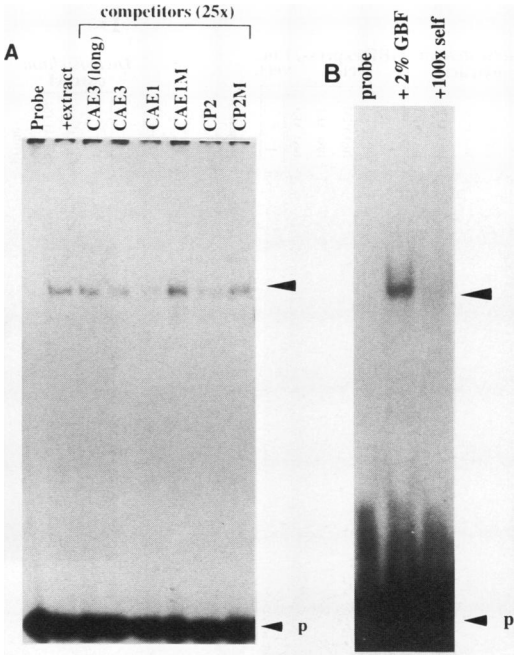

FIG. 6. *SP60/cotC* CAE-binding activity in nuclear and partially purified GBF extracts. (A) The 54-bp CAE3 oligonucleotide was labelled and incubated with proteins in a developmental nuclear extract fractionated by precipitation between 20 and 33% ammonium sulfate. Binding was inhibited with a 25-fold molar excess of the unlabelled oligonucleotides 54-bp CAE3 [CAE3 (long)], 30-bp CAE3 (CAE3), CAE1, mutant CAE1 (CAE1M), CP2 G box (CP2), and mutant CP2 G box (CP2M). Reactions were assayed by electrophoresis on 4% polyacrylamide gels. The position of free probe is indicated by an arrowhead labelled p. Another arrowhead indicates the position of the complex formed. (B) Labelled 30-mer CAE3 oligonucleotide was incubated with GBF that had been partially purified by DNA-cellulose and affinity column chromatography (~2% GBF, by mass of protein). The complex formed was inhibited with unlabelled CAE3 oligonucleotide.

DISCUSSION

In this study, we have analyzed the *SP60/cotC* promoter and have delimited the regulatory sequences sufficient to confer cell-type-specific expression on a heterologous promoter. This region contains a novel AT-rich element (or elements), the CA-rich repeat CAE3, and neighboring sequences that act synergistically to induce prespore-specific transcription during multicellular development. The *SP60/cotC* CAEs bind a cAMP-induced, developmentally regulated nuclear activity in a sequence-specific manner. We have identified this activity as GBF.

GBF activates prespore-specific transcription. Full induction of the *SP60/cotC* promoter requires all three CAEs. Point mutations or small deletions in any of the *SP60/cotC* CAEs decrease promoter activity 10- to 25-fold, and if all three are removed in a 5′ deletion, promoter expression is not detectable. Thus, while *SP60/cotC* promoter induction requires at least one CAE, any one of the three can be deleted, suggesting some redundancy in their function (16). We have shown here that a 95-bp *SP60/cotC* promoter region containing CAE3 is

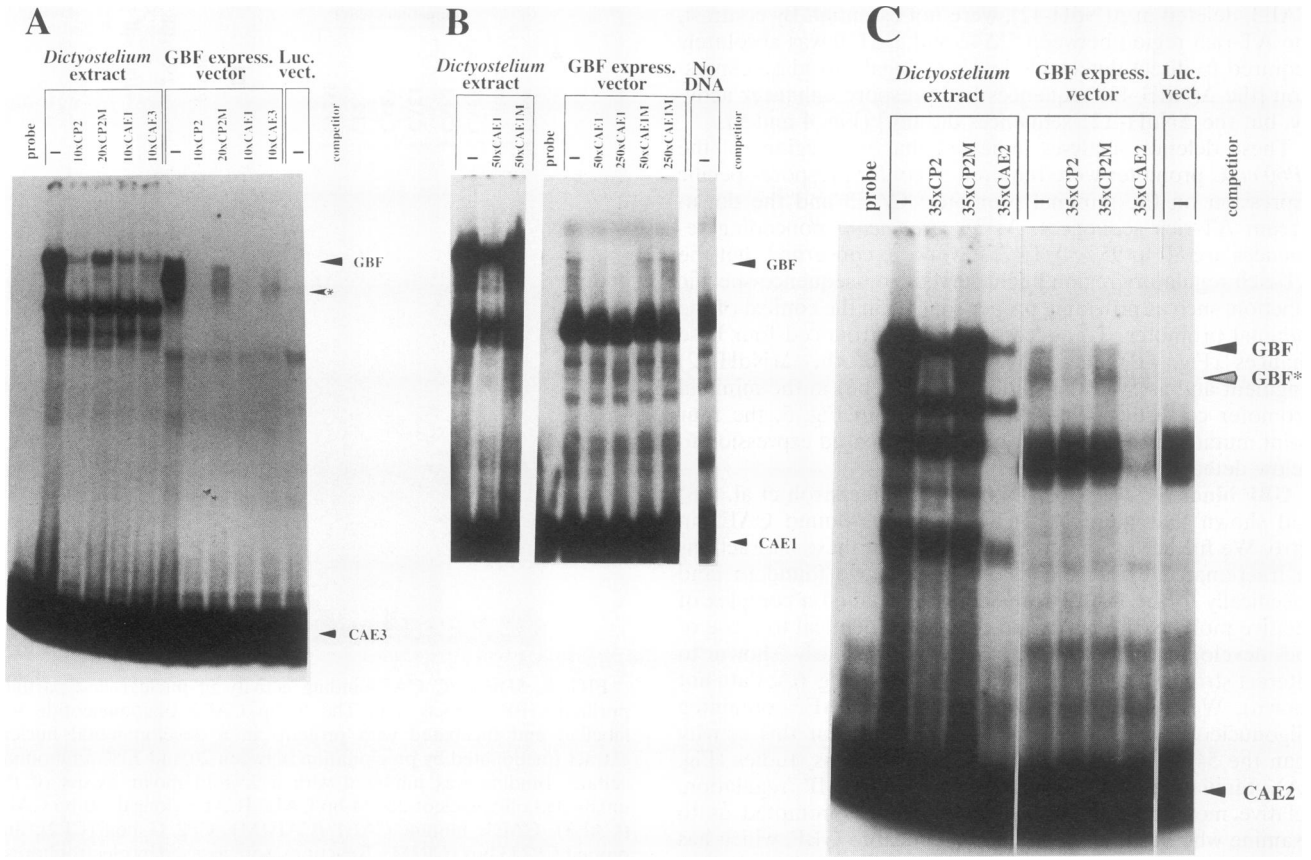

FIG. 7. *SP60/cotC* CAEs bind in vitro-transcribed and -translated GBF in a sequence-specific manner. (A) Labelled 30-mer CAE3 probe was incubated with either no extract (probe lane), a *Dictyostelium* extract enriched for GBF activity by nonspecific-DNA-cellulose affinity chromatography, a CP2-G-box-affinity-purified in vitro transcription-translation reaction mixture programmed with a GBF expression vector, or an equivalent amount of affinity-purified control reaction mixture programmed with a luciferase vector (Luc. vect.). Binding buffer was 120 mM ACB, and only 50 ng of poly(dI-dC) and 150 ng of poly(dA-dT) were included as nonspecific competitors (see Materials and Methods). Unlabelled oligonucleotides were added at the listed molar excesses to compete for binding. For competitor names and conditions, see the legend to Fig. 6. The positions of the free probe (CAE3) and the full-length GBF complex (GBF) are indicated with arrowheads. The arrowhead with an asterisk marks the position of a lower-migrating band that is specifically inhibited and present only in the GBF-programmed lysate and probably represents truncated GBF produced in the in vitro transcription-translation system. (B) As in part A, except that CAE1 was used as probe. Two microliters of unpurified transcription-translation reaction mixtures was used, and the control reaction mixture contained no vector DNA. Poly(dI-dC) (200 ng) was used as nonspecific competitor, and the binding buffer was 10 mM Tris (pH 8.5)–80 mM KCl–20 μM EDTA–200 μM dithiothreitol. (C) As in part B, except that CAE2 was used as a probe. Three microliters of unpurified GBF and/or luciferase control reaction mixtures was incubated with 0.1 ng of labelled CAE2 oligonucleotide (37 bp). The reaction buffer was 100 mM ACB, with 100 ng of poly(dI-dC) and 150 ng of poly(dA-dT) included as nonspecific competitors. The shaded arrowhead marks the position of a lower-migrating band that is specifically inhibited and present only in the GBF-programmed lysate and probably represents truncated GBF produced in the in vitro transcription-translation system.

sufficient (when multimerized) to confer prespore-specific expression on a heterologous promoter, and that CAE3 is an essential component of these sequences. While the 25 bp immediately downstream of CAE3 can be removed [in Δ(NdH-42)] without dramatically reducing the activity of these regulatory sequences, partial deletion of CAE3 [in Δ(24-64)] reduces expression to below detectable levels.

Haberstroh et al. (16) demonstrated that a developmentally regulated activity bound CAE1 and CAE2 in a sequence-specific manner. We expanded on this previous work and have now shown that CAE3, which can function with the AT element to confer prespore-specific expression on a minimal promoter, also binds this activity, although with lower affinity. Several data presented in this report demonstrate that this activity is GBF. First, GBF (bound to the CP2 G box) and the CAE-binding activity have similar competition profiles in crude or partially purified *D. discoideum* extracts. In fact, CAE1 has as high an affinity for GBF as any known binding site. Second, the CAE-binding activity copurifies with GBF. Third, in vitro-transcribed and -translated GBF binds CAE1, CAE2, and CAE3 individually to form a complex that migrates at a position in a gel retardation assay similar to that of the complex formed with *D. discoideum* extracts. In some experiments (Fig. 7), the complex formed by in vitro-transcribed and -translated GBF and CAE3 has a slightly faster mobility than the complex formed in *D. discoideum* extracts, possibly because of posttranslational modifications of GBF in vivo. Furthermore, the recombinant GBF has the same competition profile as the CAE-binding activity in nuclear extracts. We conclude that the CAE-binding activity is GBF.

GBF has been shown previously to interact with G/T- or C/A-rich sequences present in multiple promoters, including elements that mediate the transcriptional activation of the prestalk genes *CP2/pst-cathepsin* and *ecmB* (3, 19, 20). We have

shown here, for the first time, that GBF also plays a direct and essential role in the induction of prespore gene expression, through interactions with the CAEs. Because the binding affinity of CAE3 for GBF is relatively low, we considered that CAE3 might function through interaction with DNA-binding factors other than GBF. However, we assayed fractionated extracts and were unable to detect any other activity that bound CAE3 in a sequence-specific manner. Therefore, we believe that all three *SP60/cotC* CAEs function as GBF binding sites.

Deletions removing both CAE1 and CAE2 have been shown to decrease promoter activity substantially more than the sum of the respective decreases due to deletions removing the two elements individually (16). Haberstroh et al. (16) noted that this was suggestive of cooperative interactions between the complexes that bound the *SP60/cotC* CAEs. Consistent with this model, we find here that a single copy of the Δ13-Δ21 sequences does not confer detectable levels of expression on the minimal promoter construct. However, when these sequences are multimerized, such that there are three copies of CAE3, the AT element, and flanking sequences, they direct high levels of prespore-specific transcription.

Previous analyses of CAEs indicated that GBF binding sites function in a context-dependent manner. If CAE1 is deleted or mutated, the remaining *SP60/cotC* promoter sequences direct higher levels of expression in the anterior region of the prespore zone than in the posterior region. In contrast, deletion of CAE3 results in a subtle posterior-to-anterior gradient of expression. Point mutations in CAE2 do not detectably change the expression pattern (15, 16). These data suggest a model in which the induction of the *SP60/cotC* promoter is downstream of at least two signalling pathways that are activated in opposing gradients in the prespore zone (16). Thus, the three GBF binding sites, in the context of the full *SP60/cotC* promoter, may mediate responses to different signals.

The smallest *SP60/cotC* promoter fragments that specify prespore-specific expression lack CAE1 and CAE2, yet these constructs direct expression uniformly throughout the prespore zone (Fig. 4 and 5). This may indicate that sequences downstream of the AT element(s) mediate the anterior-to-posterior graded activation reported by Haberstroh et al. (16) in 5′ deletion constructs lacking the distal CAEs. We showed that internal deletion of the 105 bp between 3′Δ13 and 5′Δ56 decreases expression 200-fold. While these sequences are not absolutely required for cell-type-specific expression, associated nuclear factors may interact with complexes on CAE3 and the AT element(s) to mediate activation in response to graded signals. A second possibility is that multimerization of the *SP60/cotC* sequences in the *Act15* promoter context influences the expression pattern within the prespore region. The absence of a graded response, however, does not detract from the conclusion that CAE3, the AT-element(s), and adjacent flanking sequences include all the *cis*-acting sequences sufficient for prespore-specific transcriptional activation. However, in the context of the full promoter, a deletion of CAE3 does not destroy gene expression or prespore specificity, which may indicate that any CAE, in conjunction with the AT region, may direct prespore-specific expression.

Novel AT element is essential for enhancement of prespore transcription. While a GBF binding site is required for *SP60/cotC* promoter activation, it is not sufficient. The AT-rich sequences between 3′Δ110 (−318 bp) and 5′Δ42 (−355 bp) are also necessary to direct expression in prespore cells. The 343 bp upstream of 3′Δ110 confer high levels of prespore-specific expression on the *Act15* minimal promoter. However, a 10-bp 3′ deletion of these sequences to 3′Δ111 decreases expression to below detectable levels, even though CAE1, CAE2, and CAE3 are left intact. None of the constructs that lack these AT-rich sequences are capable of directing prespore expression. The sequence-specific function of this element (or elements) is confirmed by the introduction of four point mutations into the AT region of the Act15-60-3Δ(NdH-42) construct. While the original construct directs prespore-specific expression, the point mutations reduce expression to below detectable levels.

In a concurrent analysis of the *PspB* promoter, we identified a 46-bp AT-rich region that is essential for high levels of cell-type-specific expression (37). Comparison of the *SP60/cotC* and *PspB* AT elements revealed an extensive consensus sequence (Fig. 4B). This suggests that these sequences may interact with common regulatory factors that enhance the expression of these genes in prespore cells. Preliminary data indicate that there are nuclear activities in fractionated extracts that bind the *SP60/cotC* AT element in the presence of 0.5 μg of poly(dA-dT). Some of these interactions are sequence specific: the wild-type element competes for binding, but the b3 mutant does not (36). Studies to determine the specificity and regulation of these nuclear factors are currently under way.

Implications for the regulation of prespore differentiation. The data presented here, taken with the analyses of Hjorth et al. (20), indicate that GBF functions in both prestalk and prespore cells. To confirm this, we separated prestalk and prespore cell populations and determined that *GBF* mRNA and GBF activity are present in both cell types, with an approximately 2.5-fold enrichment in prespore cells (39; also data not shown). Recently, we also demonstrated that *gbf* null cells cannot be induced to express any of the late genes and do not execute the developmental program (39). Thus, GBF is required for both prestalk- and prespore-specific gene expression, but it is likely that it must interact with other nuclear factors to activate these late genes.

In this study, we have shown that the GBF binding site CAE3 cannot confer detectable levels of developmental activation on the *Actin15* minimal promoter, even when multimerized. Another regulatory sequence, the AT element(s), is also required for prespore-specific expression. Thus, the activation of promoters in prespore cells may require a cooperative interaction between GBF and nuclear factors that recognize the AT element(s). Further characterization of the nuclear activities that interact with the AT element(s) will enable us to investigate this hypothesis. We might expect that the AT-element binding factors are expressed only in prespore cells or that the element may interact with prespore-specific variants of GBF.

While we have shown that GBF binds G boxes and prespore CAEs (37, 39) (Fig. 7), we are still investigating the extent to which GBF binding sites are functionally equivalent. In preliminary studies, we have found that both CAE1 and CAE3 can replace the G box in the *CP2/pst-cathepsin* promoter to direct prestalk-enriched transcription (1). This suggests that the conserved region of the CAE sequences can interact with GBF or GBF complexes in prestalk cells. However, we cannot conclude from this that all GBF binding sites could, in association with the AT element, direct prespore-specific transcription. Before we can address this question definitively, we must first determine the sequences within or immediately adjacent to the CAEs and the G boxes that define their respective binding properties. Once this is accomplished, we can distinguish the functions of the binding sites themselves from those of neighboring elements.

The sequence of the *SP60/cotC* CAE flanking sequences might specify interaction with prespore-specific GBF complexes. Such complexes might be distinguished by posttranslational modifications of GBF or by association with prespore-specific factors. The two-half-site nature of the GBF binding sites (20) suggests that it binds DNA as a dimer. While GBF can bind the CP2 G box (39) or any of the three *SP60/cotC* CAEs (this report) in the absence of heterodimeric partners in vitro, it may form functionally distinct heterodimers in vivo. Given the prevalence of heterodimer formation as a mechanism of transcriptional regulation in other systems (23, 24, 27), we are investigating this possibility. Now that GBF has been purified and cloned, the identification of GBF-associated proteins is feasible.

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REFERENCES

1. **Cao, J., J. A. Powell-Coffman, C. Gaskins, and R. A. Firtel.** Unpublished observations.
2. **Ceccarelli, A., H. Mahbubani, and J. G. Williams.** 1991. Positively and negatively acting signals regulating stalk cell and anterior-like cell differentiation in *Dictyostelium*. Cell **65**:983–989.
3. **Ceccarelli, A., H. J. Mahbubani, R. Insall, G. Schnitzler, R. A. Firtel, and J. G. Williams.** 1992. A G-rich sequence element common to *Dictyostelium* genes which differ radically in their patterns of expression. Dev. Biol. **152**:188–193.
4. **Chisholm, R. L., E. Barklis, and H. F. Lodish.** 1984. Mechanism of sequential induction of cell-type specific mRNAs in *Dictyostelium* differentiation. Nature (London) **310**:67–69.
5. **Cubitt, A. B., F. Carrel, S. Dharmawardhane, C. Gaskins, J. Hadwiger, P. Howard, S. K. O. Mann, K. Okaichi, K. Zhou, and R. A. Firtel.** 1992. Molecular genetic analysis of signal transduction pathways controlling multicellular development in *Dictyostelium*. Cold Spring Harbor Symp. Quant. Biol. **LVII**:177–192.
6. **Datta, S., and R. A. Firtel.** 1988. An 80-bp cis-acting regulatory region controls cAMP and development regulation of a prestalk gene in *Dictyostelium*. Genes Dev. **2**:294–304.
7. **Dingermann, T., N. Reindl, H. Werner, M. Hildebrandt, W. Nellen, A. Harwood, J. Williams, and K. Nerke.** 1989. Optimization and in situ detection of Escherichia coli beta-galactosidase gene expression in *Dictyostelium discoideum*. Gene **85**:353–362.
8. **Early, A., and J. Williams.** 1988. A *Dictyostelium* prespore-specific gene is transcriptionally repressed by DIF in vitro. Development **103**:519–524.
9. **Early, A. E., M. J. Gaskell, C. Traynor, and J. G. Williams.** 1993. Two distinct populations of prestalk cells within the tip of the migratory *Dictyostelium* slug with differing fates at culmination. Development **118**:353–362.
10. **Early, A. E., and J. G. Williams.** 1989. Identification of sequences regulating the transcription of a *Dictyostelium* gene selectively expressed in prespore cells. Nucleic Acids Res. **17**:6473–6484.
11. **Fosnaugh, K. L., and W. F. Loomis.** 1991. Coordinate regulation of the spore coat genes in *Dictyostelium discoideum*. Dev. Genet. **12**:123–132.
12. **Fosnaugh, K. L., and W. F. Loomis.** 1993. Enhancer regions responsible for temporal and cell-type-specific expression of a spore coat gene in *Dictyostelium*. Dev. Biol. **157**:38–48.
13. **Gomer, R., and R. Firtel.** 1987. Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell cycle phase. Science **237**:758–762.
14. **Gomer, R. H., S. D. Datta, and R. A. Firtel.** 1986. Cellular and subcellular distribution of a cAMP-regulated prestalk protein and prespore protein in *Dictyostelium discoideum*. J. Cell Biol. **103**: 1999–2015.
15. **Haberstroh, L., and R. A. Firtel.** 1990. A spatial gradient of expression of a cAMP-regulated prespore cell-type-specific gene in *Dictyostelium*. Genes Dev. **4**:596–612.
16. **Haberstroh, L., J. Galindo, and R. A. Firtel.** 1991. Developmental and spatial regulation of a *Dictyostelium* prespore gene: cis-acting elements and a cAMP-induced, developmentally regulated DNA binding activity. Development **113**:947–958.
17. **Hadwiger, J. A., and R. A. Firtel.** 1992. Analysis of Gα4, a G-protein subunit required for multicellular development in *Dictyostelium*. Genes Dev. **6**:38–49.
18. **Hjorth, A., S. Datta, N. Khanna, and R. Firtel.** 1988. Analysis of cis and trans elements involved in cAMP-inducible gene expression in *Dictyostelium discoideum*. Dev. Genet. **9**:435–454.
19. **Hjorth, A. L., N. C. Khanna, and R. A. Firtel.** 1989. A trans-acting factor required for cAMP-induced gene expression in *Dictyostelium* is regulated developmentally and induced by cAMP. Genes Dev. **3**:747–759.
20. **Hjorth, A. L., C. Pears, J. G. Williams, and R. A. Firtel.** 1990. A developmentally regulated trans-acting factor recognizes dissimilar G/C-rich elements controlling a class of cAMP-inducible *Dictyostelium* genes. Genes Dev. **4**:419–432.
21. **Hopper, N. A., C. Anjard, C. D. Reymond, and J. G. Williams.** 1993. Regulation of terminal differentiation of *Dictyostelium* by PKA and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. Development **119**:147–154.
22. **Howard, P. K., K. G. Ahern, and R. A. Firtel.** 1988. Establishment of a transient expression system for *Dictyostelium discoideum*. Nucleic Acids Res. **16**:2613–2623.
23. **Jacob, S. T., and R. R. Reichel.** 1993. Control of gene expression by lipophilic hormones. FASEB J. **7**:427–436.
24. **Jones, N.** 1990. Transcriptional regulation by dimerization: two sides to an incestuous relationship. Cell **61**:9–11.
25. **Kimmel, A. R., and R. A. Firtel.** 1982. The organization and expression of the *Dictyostelium* genome, p. 234–324. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
26. **Krefft, M., L. Voet, J. Gregg, H. Mairhofer, and K. Williams.** 1984. Evidence that positional information is used to establish the prestalk-prespore pattern in *Dictyostelium discoideum* aggregates. EMBO J. **3**:201–206.
27. **Lee, K. A.** 1992. Dimeric transcription factor families: it takes two to tango but who decides on partners and venue? J. Cell Sci. **103**:9–14.
28. **Loomis, W. F. (ed.).** 1982. The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
29. **Mann, S. K., and R. A. Firtel.** 1991. A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*. Mech. Dev. **35**:89–101.
30. **Mann, S. K., W. M. Yonemoto, S. S. Taylor, and R. A. Firtel.** 1992. DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **89**:10701–10705.
31. **Mann, S. K. O., and R. A. Firtel.** 1993. cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during *Dictyostelium* development. Development **119**:135–146.
32. **Mehdy, M., and R. Firtel.** 1985. A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*. Mol. Cell. Biol. **5**:705–713.
33. **Mehdy, M. C., D. Ratner, and R. A. Firtel.** 1983. Induction and modulation of cell type specific gene expression in *Dictyostelium*. Cell **32**:763–771.
34. **Ozaki, T., H. Nakao, H. Orii, T. Morio, I. Takeuchi, and M. Tasaka.** 1993. Developmental regulation of transcription of a novel prespore-specific gene (Dp87) in *Dictyostelium discoideum*. Development **117**:1299–1308.
35. **Pears, C. J., and J. G. Williams.** 1988. Multiple copies of a G-rich element upstream of a cAMP-inducible *Dictyostelium* gene are necessary but not sufficient for efficient gene expression. Nucleic Acids Res. **16**:8467–8486.
36. **Powell-Coffman, J. A., and R. A. Firtel.** Unpublished observations.
37. **Powell-Coffman, J. A., and R. A. Firtel.** Characterization of a novel

Dictyostelium discoideum prespore-specific gene, *PspB*, reveals conserved regulatory sequences. Development, in press.

38. **Schaap, P., M. M. Van Lookeren Campagne, R. Van Driel, W. Spek, P. J. Van Haastert, and J. Pinas.** 1986. Postaggregative differentiation induction by cyclic AMP in *Dictyostelium*: intracellular transduction pathway and requirement for additional stimuli. Dev. Biol. **118**:52–63.
39. **Schnitzler, G. R., W. H. Fischer, and R. A. Firtel.** 1994. Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in *Dictyostelium*. Genes Dev. **8**:502–514.
40. **Tasaka, M., M. Hasegawa, M. Nakata, H. Orii, T. Ozaki, and I. Takeuchi.** 1992. Protein binding and DNase-I-hypersensitive sites in the cis-acting regulatory region of the spore-coat SP96 gene of *Dictyostelium*. Mech. Dev. **36**:105–115.
41. **Weijer, C., G. Duschl, and C. David.** 1984. Dependence of cell-type proportioning and sorting on cell cycle phase in *Dictyostelium discoideum*. J. Cell Sci. **70**:133–146.
42. **Williams, J. G., K. T. Duffy, D. P. Lane, S. J. McRobbie, A. J. Harwood, D. Traynor, R. R. Kay, and K. A. Jermyn.** 1989. Origins of the prestalk-prespore pattern in *Dictyostelium* development. Cell **59**:1157–1163.