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Functional and Regulatory Analysis of the *Dictyostelium* G-Box Binding Factor

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The *Dictyostelium discoidium* G-box binding factor (GBF) is required for the induction of known postaggregative and cell-type-specific genes. *gbf*-null cells undergo developmental arrest at the loose-mound stage due to the absence of GBF-targeted gene transcription. GBF-mediated gene expression is activated by stimulation of cell-surface, seven-span cAMP receptors, but this activation is independent of heterotrimeric G-proteins. To further characterize GBF, we assayed a series of GBF mutants for their ability to bind a G-box *in vitro* and to complement the *gbf*-null phenotype. *In vitro* DNA-binding activity resides in the central portion of the protein, which contains two predicted zinc fingers. However, *in vivo* GBF function requires only one intact zinc finger. In addition, expression of some GBF mutants results in a partial complementation phenotype, suggesting that these mutants are hypomorphic alleles. We used a 2.4-kb GBF-promoter fragment to examine the regulation of GBF expression. GBF promoter-reporter studies confirmed the previous finding that GBF transcription is induced by continuous, micromolar extracellular cAMP. We also show that, like the activation of GBF-regulated transcription, the induction of GBF expression requires cell-surface cAMP receptors, but not heterotrimeric G-proteins. Finally, reporter studies demonstrated that induction of GBF-promoter-regulated expression does not require the presence of GBF protein, indicating that GBF expression is not regulated by a positive autoregulatory loop. © 2001 Academic Press

Key Words: Dictyostelium; **transcription factor; gene regulation.**

INTRODUCTION

Under starvation conditions, up to ~10^5 *Dictyostelium* cells aggregate to create a multicellular organism. The formation of a mound of cells is followed by cell-type differentiation and morphogenesis to eventually yield a fruiting body containing a mass of dormant spores supported by a long, slender stalk (Aubry and Firtel, 1999; Firtel, 1995; Loomis and Cann, 1982; Williams, 1995). A developmental transition occurs as cells are completing aggregation. Increasing concentrations of extracellular cAMP (Abe and Yanagisawa, 1983) and other signals mediate the down-regulation of gene products required for aggregation and the induction of a variety of genes essential for postaggregative morphogenesis and cell-type differentiation (Mann and Firtel, 1987; Mehdy and Firtel, 1985; Schaap and van Driel, 1985; Town and Gross, 1978). Suspension assays designed to simulate multicellular development *in vitro* show that the expression of one class of these genes, the postaggregative genes (e.g., *LagC*, *CP2*), requires only a period of starvation followed by stimulation with high, continuous cAMP, so that cell-surface receptors remain saturated (Dynes *et al.*, 1994; Mehdy and Firtel, 1985; Mehdy *et al.*, 1983; Reymond *et al.*, 1984). Prestalk-specific (e.g., *ecmA*, *ecmB*) and prespore-specific (e.g., *SP60/cotB*, *SP70/cotC*) cells require cell–cell contacts and the morphogen DIF or extracellular cAMP, respectively (Aubry and Firtel, 1999; Berks and Kay, 1990; Fosnaugh and Loomis, 1991; Mehdy and Firtel, 1985; Mehdy *et al.*, 1983; Williams, 1995; Williams *et al.*, 1987). The expression of all of these genes is dependent on GBF, a highly basic transcription factor containing two predicted zinc fingers that bind to the G-box, a conserved promoter element found upstream of each gene (Dynes *et al.*, 1994; Hjorth *et al.*, 1989, 1990; Schnitzler *et al.*, 1994, 1995). Promoter mutational analyses demonstrated a direct correlation between the ability to bind GBF *in vitro* and promoter activity *in vivo* (Ceccarelli

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et al., 1991; Datta and Firtel, 1987; Datta *et al.*, 1987; Esch *et al.*, 1992; Fosnaugh and Loomis, 1993; Haberstroh and Firtel, 1990; Haberstroh *et al.*, 1991; Pears and Williams, 1987; Powell-Coffman and Firtel, 1994).

Cells in which the GBF locus is disrupted (*gbf*-null cells) undergo developmental arrest at the loose-mound stage and do not express postaggregative or cell-type-specific genes, either in developing cells or when stimulated with cAMP in suspension assays (Schnitzler *et al.*, 1994). Expression of GBF from the constitutive Actin 15 (Act15) promoter in *gbf*-null cells complements the developmental phenotype. However, although GBF protein isolated from vegetative cells of this strain is able to bind G-boxes in an electrophoretic mobility shift assay, induction of GBF-target genes is dependent on stimulation with extracellular cAMP (Brown *et al.*, 1997; Schnitzler *et al.*, 1995). Overexpression of GBF in a variety of strains containing gene disruptions in signaling components suspected of being involved in cAMP-stimulated GBF activity reveals that cell-surface cAMP receptors (cAR1 or cAR3) are required for function (Schnitzler *et al.*, 1995). Surprisingly, GBF activation does not require the only known Gβ-subunit found in *Dictyostelium* (Lilly *et al.*, 1988; Wu *et al.*, 1995) or the Gα subunit Gα2 (Kumagai *et al.*, 1991), which is coupled to cAR1 during aggregation and mediates activity of adenylyl cyclase and chemotaxis, indicating that the GBF pathway is G-protein-independent (Schnitzler *et al.*, 1995). Other G-protein-independent events are stimulated by increased cAMP at the mound stage as well, including the phosphorylation and nuclear translocation of the *Dictyostelium* STAT homologue *DdSTATa* (Araki *et al.*, 1998; Kawata *et al.*, 1997).

To further characterize the function of GBF, we have created a series of deletion mutants of the protein. This analysis has identified regions of the protein required for either DNA binding *in vitro* or *in vivo* complementation of the *gbf*-null phenotype. Expression of some mutant proteins in *gbf*-null cells results in partial complementation, suggesting that these hypomorphic alleles may preferentially activate only a subset of GBF-target genes. We found, unexpectedly, that GBF function requires only one intact zinc finger and that either zinc finger will suffice. In addition, we have examined the regulation of GBF expression. GBF is present at low levels in vegetative cells and, like GBF-target genes, is rapidly up-regulated upon stimulation with micromolar cAMP. This led to the proposal that GBF induces its own high-level expression via an autoactivation loop. We have isolated the GBF promoter and used *lacZ* and luciferase fusions to demonstrate that the presence of GBF protein is not required for the normal induction of GBF promoter activity. Finally, we have measured the activity of the GBF promoter in a variety of mutant backgrounds and found that, like the cAMP-mediated stimulation of GBF activity, GBF promoter induction is cAMP-receptor-dependent but does not require Gβ. Results from additional experiments indicate that GBF induction requires neither intracellular cAMP nor LagC, a cell-surface molecule previously shown to play a role in the maintenance of GBF expression during the later stages of multicellular development (Sukumaran *et al.*, 1998).

MATERIALS AND METHODS

Cell Culture Conditions and Transformation of Dictyostelium Cells, lacZ Staining, and General Molecular Biology

We used the axenic strain KAx-3 (Mann and Firtel, 1991) as a wild-type strain for all experiments except those that required auxotropic selection with thymidine, in which we used JH10 (Hadwiger and Firtel, 1992). Methods for *Dictyostelium* culture, development, DNA or RNA isolation (Nellen *et al.*, 1987), electroporation (Dynes and Firtel, 1989), *lacZ* staining (Dingermann *et al.*, 1989; Haberstroh and Firtel, 1990; Powell-Coffman and Firtel, 1994), fast- and slow-shake experiments (Mehdy *et al.*, 1983; Mehdy and Firtel, 1985), and hybridization (Powell *et al.*, 1992) have been previously described.

Construction of GBF Mutants

A double-stranded oligonucleotide containing a 6X His tag followed by a *Bam*HI site was inserted into the *Eco*RI site just downstream of the GBF start codon in the R20 plasmid (Schnitzler *et al.*, 1994). We created N-terminal deletion mutants by exonuclease III digestion after linearizing with *Bam*HI. We made C-terminal deletions with appropriate stop codons by PCR and confirmed them by DNA sequencing. Point mutations were created by using the Transformer Site-Directed Mutagenesis Kit (Clontech). We constitutively expressed mutant cDNAs in *Dictyostelium* using the extrachromosomal expression vector DEP-j (K. Zhou and R.A.F., unpublished observations).

Extract Preparation and Mobility Shift Assays

We produced cytoplasmic extracts as previously described (Schnitzler *et al.*, 1994). We performed mobility shift assays as described previously (Schnitzler *et al.*, 1994) with the following modifications. Each 20-μl reaction contained 0.15 ng labeled CP2 43-mer probe (Hjorth *et al.*, 1989), 1 mg/ml BSA, 400 ng poly[dI-C] along with the appropriate volume of extract for 1 μg total protein. Unlabeled competitor DNA (5 ng), where indicated, was added prior to addition of cytoplasmic extract.

GBF Promoter Cloning

We isolated 2.4 kb of GBF upstream promoter sequence by homologous integration of the plasmid pUCBsr (Sutoh, 1993) into the GBF locus followed by plasmid rescue into *Escherichia coli* by using a previously mapped genomic *Hin*dIII site (Schnitzler *et al.*, 1994). To accomplish this, we constructed, a "head-to-tail" GBF cDNA plasmid, which, when linearized, produces the integration fragment shown in Fig. 5. First, we subcloned the 1.7-kb *Kpn*I fragment of R20 into pUCBsr so the 3′ end of the cDNA was closest to the pUC118 backbone. We used PCR to amplify a 700-bp portion of GBF cDNA closer to the 5′ end. We subcloned this fragment using PCR-generated *Eco*RI ends into the plasmid so that 1.0 kb of the GBF sequence was replaced. We performed restriction mapping

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to confirm that the 5′-most end of the PCR fragment was adjacent to the 3′-most end of the remaining *Eco*RI–*Kpn*I GBF fragment. The plasmid was linearized by using a PCR-generated *Cla*I site at the GBF 5′/3′ junction point and transformed into KAx-3 cells. We selected transformants in HL5 medium using 15 μg/ml blasticidin. We cloned resistant strains on SM plates in association with *Klebsiella aerogenes*. Clones displaying the expected *gbf*-null phenotype (Schnitzler *et al.*, 1994) were confirmed by Southern blotting. Genomic DNA was isolated from an appropriate clone, cut with *Hin*dIII, self-ligated, and transformed into *E. coli*. Plasmid DNA was isolated and confirmed by restriction mapping. The sequence of the GBF promoter is GenBank accession no. AF337815.

Luciferase Integration into the Locus

We ligated a 1.1-kb *Bgl*II–*Xba*I GBF promoter fragment (created by exonuclease III digestion), the luciferase ORF [cut out of pSP60/luciferase (Haberstroh and Firtel, 1990; Mann *et al.*, 1998) with *Spe*I–*Kpn*I], and a *Kpn*I–*Bam*HI *Dictyostelium* Actin 8 terminator fragment into pSP72 (Promega). Into this plasmid, cut with *Hin*dIII and *Xho*I, we ligated a *Hin*dIII–*Bam*HI fragment containing the Thy1 gene (Dynes and Firtel, 1989) and a 1.5-kb fragment of $GBF^{\Delta 10-115}$ cDNA with *Bam*HI and *Xho*I ends. The entire integration cassette was released with *Bgl*II and *Xho*I, transformed into JH10 cells, and selected for growth in the absence of exogenous thymidine. We confirmed clones displaying the expected *gbf*-null phenotype by Southern blotting.

Luciferase Assays

We performed all luciferase assays using the Luciferase Assay System (Promega). For each treatment, we pelleted approximately 1.5×10^7 cells and resuspended them in 100 μl 1× Cell Lysis Buffer, supplemented with 1× Complete, EDTA-free protease inhibitors (Roche) and 1 mM $NaVO_4$. We aliquoted two 10-μl volumes of each sample into a 96-well PolySorp FluoroNunc plate (Nunc) and measured light units after addition of 50 μl Luciferase Assay Substrate using an EG&G Berthold Microplate Luminometer (LB 96V). We normalized luciferase activity values by the total protein concentration of each extract as determined by Bio-Rad protein assay. We performed each experiment at least three times using at least two independent transformed populations.

Construction of a cAR1/cAR2/cAR3 Deletion Strain

To create this strain, we disrupted the *cAR2* gene in a *cAR1*/*cAR3* strain (Insall *et al.*, 1994). To construct the *cAR2* disruption cassette, a 5′ fragment of the *cAR2* cDNA was PCR amplified by using the primers GTTTTGGATCCCAGATATTATCGCACAAAG and GTTTTTCTAGACTATACCCATCATAGTTATC. A 3′ *cAR2* cDNA fragment was PCR amplified by using the primers GTTTTAAGCTTCATTTGGGGAACATCAGC and GTTTTGGTACCGAGCATTCTTTGATCTC (all primer sequences are shown 5′ to 3′). Each PCR product was digested with the appropriate restriction enzyme (underlined in primer sequence) and ligated, along with the blasticidin resistance cassette [released from pUCBsrΔBam (Sutoh, 1993) with *Xba*I and *Hin*dIII], into pSP72 (Promega Life Sciences, Madison, WI) digested with *Bam*HI and *Kpn*I. The disruption cassette, consisting of the blasticidin resistance cassette flanked by *cAR2* cDNA fragments, was released from pSP72 by digestion with *Bam*HI and *Kpn*I and electroporated into *cAR1/cAR3*-null cells. We confirmed *cAR2* disruption in blasticidin resistant colonies by Southern blot.

RESULTS

Mutational Analysis of GBF

To functionally characterize the GBF protein, we carried out a detailed mutational analysis. We constructed a collection of mutants (Fig. 1) using exonuclease III digestion, PCR, and oligonucleotide-directed mutagenesis as described in Materials and Methods. Each mutant was tested for its ability to function in an electrophoretic mobility shift assay (EMSA; Figs. 1 and 2) and to complement the *gbf*-null phenotype when expressed from the Act15 promoter (Figs. 1 and 3). Previous experiments revealed that constitutive expression of either full-length GBF or GBFΔI, which contains an internal deletion of the Gln/His-rich domain, results in a nearly wild-type phenotype (Mann *et al.*, 1997; Fig. 3). We found that GBFΔI binds DNA properly, although the size of the mobility shift is decreased due to the reduction in the size of the protein (Mann *et al.*, 1997; Fig. 2). As we show in Fig. 2, many of the deletions examined were able to bind a G-box-containing oligonucleotide *in vitro* and exhibited a reduction in mobility shift that was related to the size of the mutant protein.

Because the Gln/His domain deletion did not affect GBF function, all mutants constructed subsequently did not contain this region. Deletion of protein sequence between residues 10–77 ($GBF^{\Delta 10-77}$) did not affect the ability to complement the mound-arrest phenotype of *gbf*-null cells, although the mature fruiting bodies were slightly deformed, with the spore mass often found just below the top of the stalk (Fig. 3). However, further deletion to residue 115 resulted in a dramatic alteration in phenotype. Terminally developed *gbf*-null cells expressing $GBF^{\Delta 10-115}$ arrest as mounds with elongated tips (Fig. 3). This partial complementation suggests that, although $GBF^{\Delta 10-115}$ must be able to activate at least a subset of GBF-induced genes required for postaggregative development, it either fails to induce others or does not induce them to the correct level. Alternatively, misexpression of one or more GBF target genes could lead to secondary effects that yield the observed phenotype. To examine these possibilities, we assayed the ability of several GBF mutants to activate the expression of the GBF-regulated genes *LagC* and *CP2*. We found that, like GBFΔI, $GBF^{\Delta 10-77}$ is able to induce both target genes (Fig. 4). However, $GBF^{\Delta 10-115}$ activates *LagC*, but not *CP2* (Fig. 4). Although the level of *LagC* expression is reduced, if the level of *CP2* expression is reduced equivalently, we would have detected it by Northern blot analysis. Expression of $GBF^{\Delta 10-283}$ in *gbf*-null cells leads to a phenotype similar to that of $GBF^{\Delta 10-115}$ (data not shown), but deletion of additional residues to give $GBF^{\Delta 10-338}$ results in a complete inability to progress beyond the mound stage (Fig. 3) or induce either *LagC* or *CP2* (Fig. 4). Even though $GBF^{\Delta 10-338}$ is deleted to nearly the predicted beginning of the first zinc-

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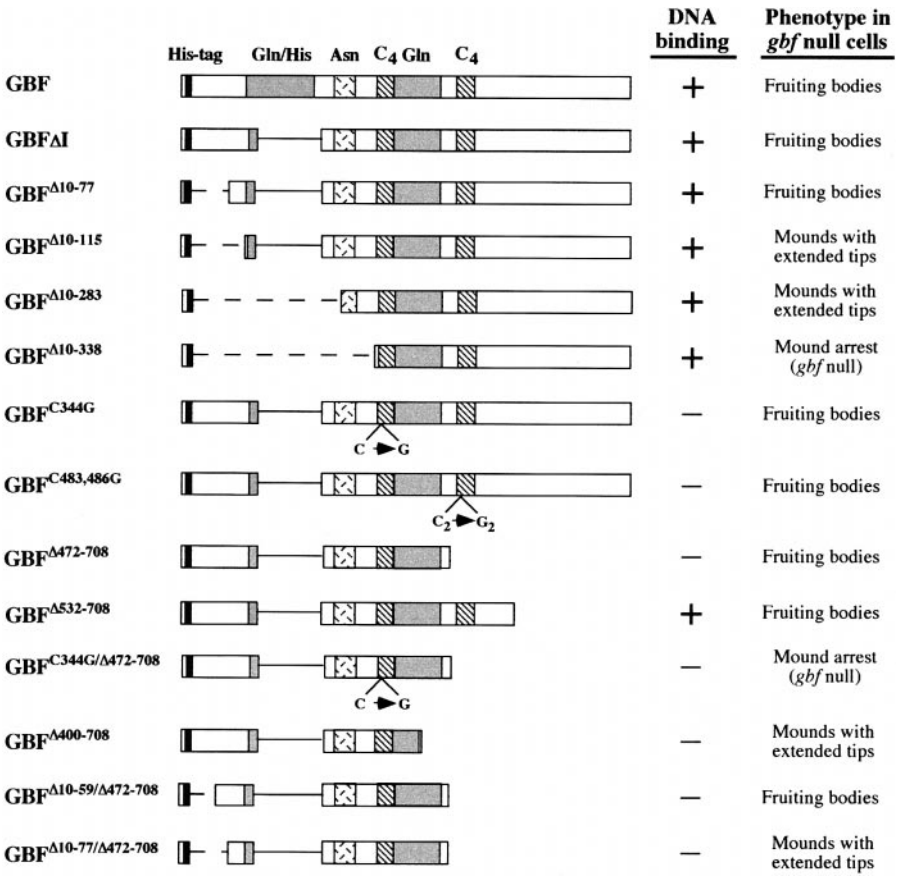

FIG. 1. Schematic representation of GBF deletion mutants. Mutants were constructed as described in Materials and Methods. The ability of each protein to bind to a 43-bp G-box-containing *CP2* promoter fragment or complement the *gbf*-null cell phenotype is summarized (see Figs. 2 and 3 for more detail).

finger domain, this protein still displays sequence-specific DNA binding in an EMSA (Fig. 2). The DNA-binding affinity of $GBF^{\Delta 10-338}$ may be reduced, as the mRNA expression level of all mutants tested was roughly equivalent (data not shown).

Deletion of protein sequence from the C terminus of GBF yielded a surprising result. Removal of the C-terminal 33% of the protein $GBF^{\Delta 472-708}$, including the second zinc finger, does not abolish the function of the protein. *gbf*-null cells expressing this mutant form fully developed, albeit somewhat smaller, fruiting bodies (Fig. 3). However, we detected no gel-shift activity in extracts made from these cells (data not shown). This was not entirely unexpected; insertion of an auxotrophic marker into the GBF locus at a site between the two zinc fingers does not result in an obvious mutant phenotype, although no GBF gel shift activity is detectable in this strain (G. R. Schnitzler and R.A.F., unpublished observations). Site-directed mutagenesis of conserved cysteine residues in either zinc-finger domain produced similar results. Constitutive expression of either an N-terminal zinc finger mutant (GBF^{C344G}; Fig. 3) or a C-terminal zinc finger mutant ($GBF^{C483,486G}$; data not shown) fully restores the ability to develop fruiting bodies. However, neither GBF^{C344G}, $GBF^{C483,486G}$, nor a combination of the two proteins is able to bind DNA *in vitro* (Fig. 2). Expression of a mutant containing a larger C-terminal deletion ($GBF^{\Delta 400-708}$) results in a partial complementation similar to that of $GBF^{\Delta 10-115}$ (Fig. 3). Like $GBF^{\Delta 10-115}$, $GBF^{\Delta 400-708}$ is able to induce the expression of *LagC* (at a reduced level), but not CP2 (Fig. 4). Deletion of a slightly smaller portion of the C terminus ($GBF^{\Delta 532-708}$) does not disrupt gel shift activity (Fig. 2). Combining the first zinc finger mutation with the large C-terminal deletion ($GBF^{C344G/\Delta 472-708}$) results in a complete inability to complement *gbf*-null cells (Fig. 3). Taken together, the results indicate that only one zinc finger domain is necessary for GBF function *in vivo* and that either zinc finger is sufficient. Mutants containing both N-terminal and C-terminal deletions suggest some functional redundancy between the two ends of the protein. Whereas $GBF^{\Delta 10-59/472-708}$ (Fig. 3) is able to form fruiting bodies, $GBF^{\Delta 10-77/472-708}$ (Fig. 3) only forms mounds with extended

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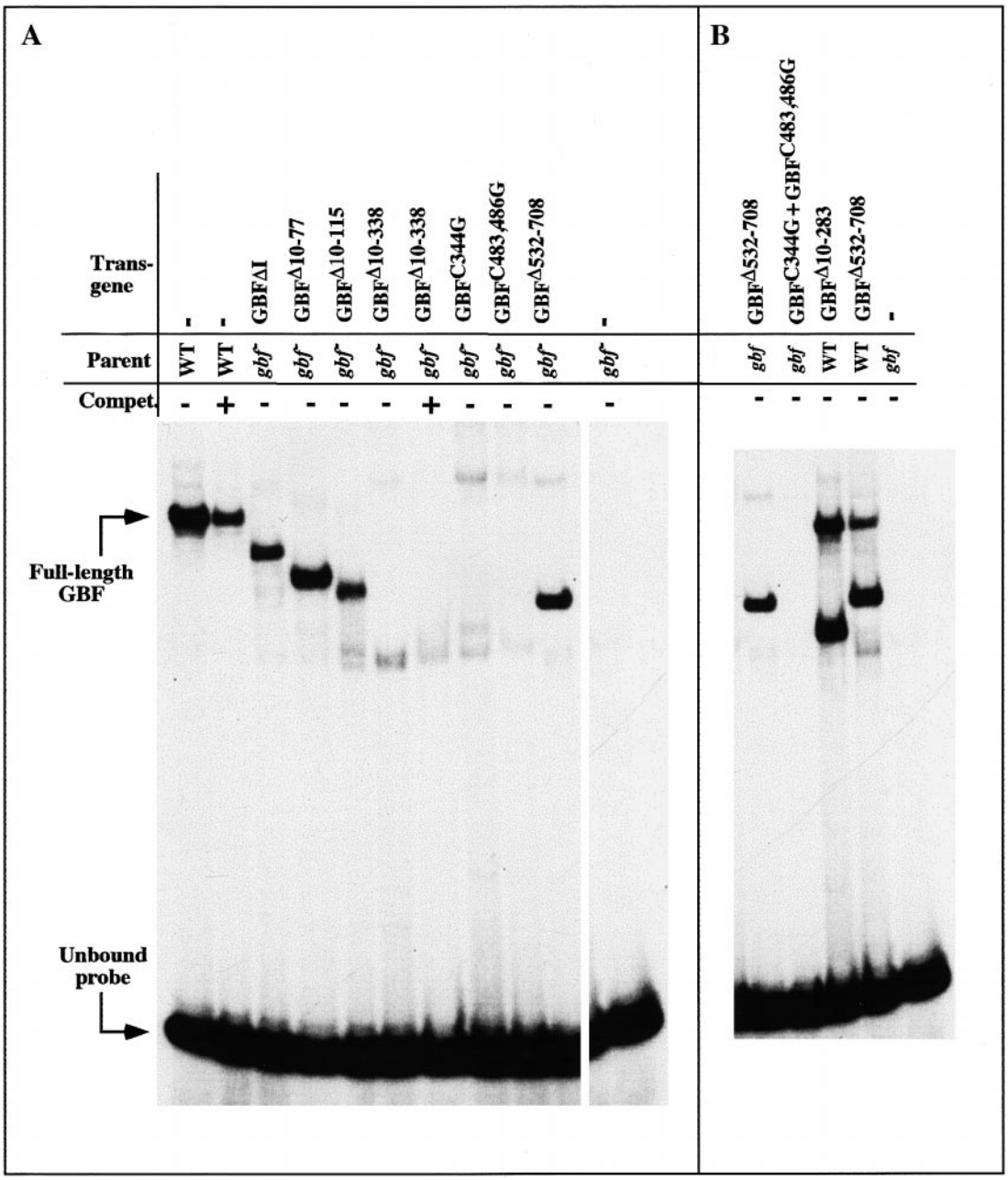

FIG. 2. Electrophoretic mobility shift assay of *GBF* deletion mutants. Cytoplasmic extracts of wild-type (KAx-3) or *gbf*-null cells constitutively expressing various GBF mutants were prepared from vegetatively growing cells. Wild-type and *gbf*-null control extracts were prepared from cells developed for 5 h. The probe contains a 43-bp portion of the *CP2* promoter that is required for GBF-regulated expression *in vivo*. Competitor reactions contain a 25-molar excess of unlabelled probe DNA. All lanes in either (A) or (B) are from the same experiment; some unrelated lanes have been removed.

tips, revealing that, in the absence of residues 472–708, amino acids 59–77 are required for terminal development.

Previous studies indicated that functional GBF binding sites require two intact DNA half-sites (Haberstroh *et al.*, 1991; Hjorth *et al.*, 1989, 1990; Pears and Williams, 1987, 1988). Given the unexpected finding that only one zinc finger is required for function *in vivo*, we examined whether GBF binds DNA as a homodimer. Cytoplasmic extracts from wild-type cells expressing either an N-terminal ($GBF^{\Delta 10-283}$) or C-terminal mutant ($GBF^{\Delta 532-708}$),

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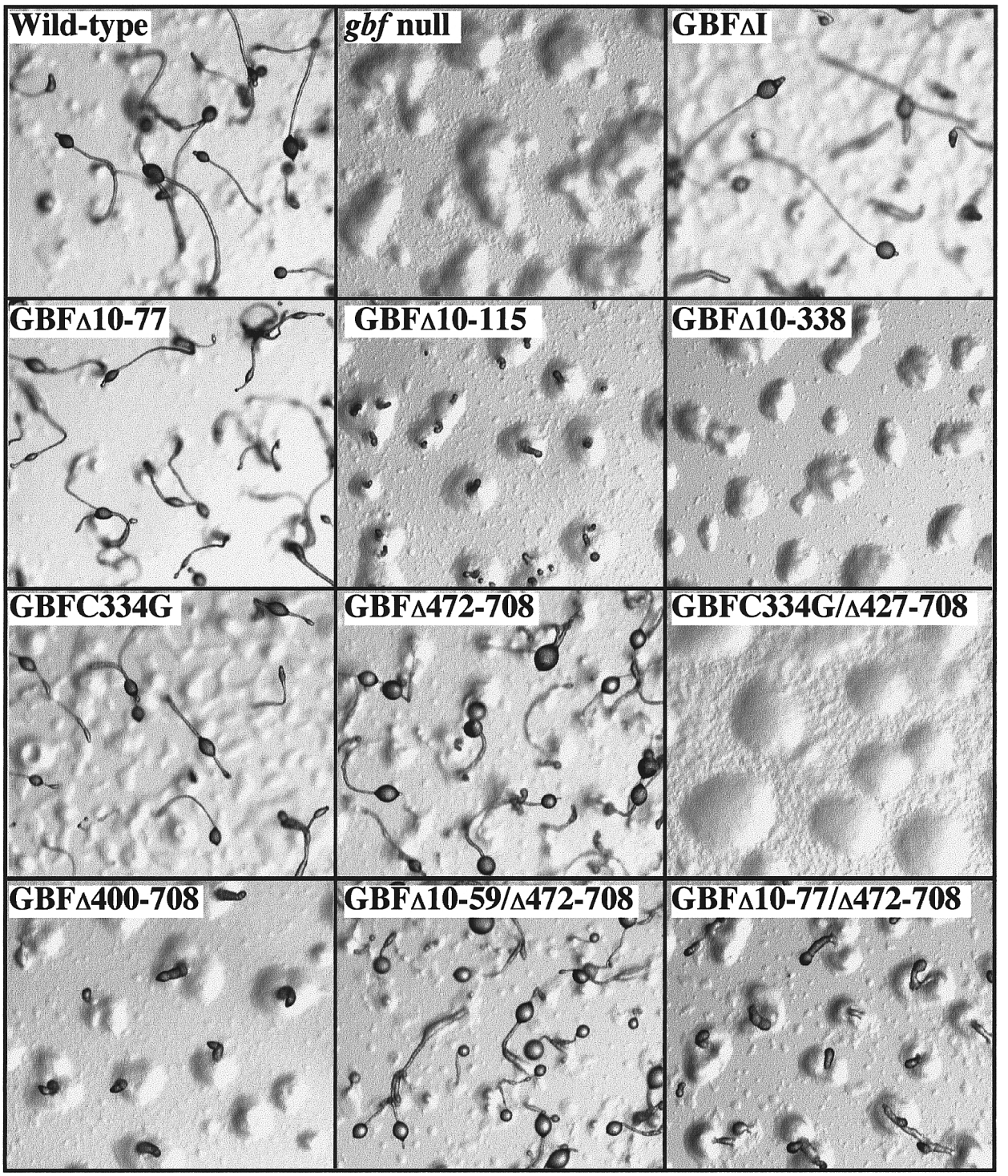

FIG. 3. Complementation of *gbf*-null cells with various deletion mutants. The terminal developmental phenotype (>26 h) of each strain is shown. All GBF mutants shown are expressed in *gbf*-null cells from the constitutive Actin 15 promoter. All pictures were taken at the same magnification.

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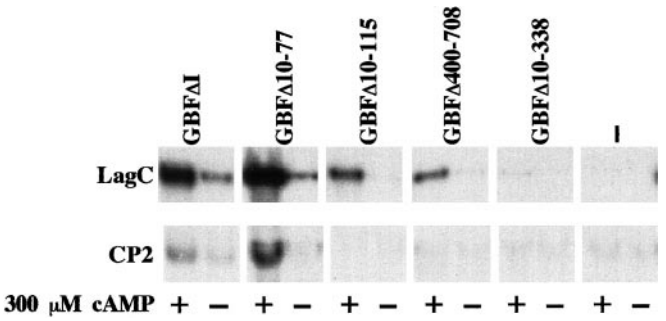

FIG. 4. GBF mutants differentially direct target gene expression. *gbf*-null cells expressing mutant GBF proteins (as indicated) that are able to complement the mound-arrest phenotype to various extents (see text for details) were examined for the ability to induce *LagC* and *CP2*. Each strain was washed free of nutrients and starved in suspension for 30 min. The cultures were then split and treated (+) or not treated (−) with 300 μM cAMP for 90 min. Total mRNA was harvested, size separated, and probed with *LagC* and *CP2*. An rRNA band that hybridizes nonspecifically with *CP2* mRNA can be distinguished by its equal intensity in the presence or absence of cAMP.

each of which is capable of binding DNA, were examined by EMSA. In both cases, we observed a band of the expected size for both full-length GBF and the constitutively expressed mutant (Fig. 2). However, we detected no bands of intermediate size, which would signify the formation of dimeric molecules.

Isolation of the GBF Promoter

The previous observation that *GBF* mRNA levels and DNA-binding activity are very low during vegetative growth and early aggregation and then increase rapidly upon mound formation or *in vitro* stimulation with cAMP suggested that GBF expression may be regulated by an auto-induction loop (Hjorth *et al.*, 1989; Schnitzler *et al.*, 1994). To test this hypothesis, we wished to compare the activity of the *GBF* promoter in wild-type and *gbf*-null cells. We isolated 2.4 kb of sequence upstream of the GBF start codon using the approach diagrammed in Fig. 5 and described in Materials and Methods. Studies using purified prestalk and prespore cell populations previously revealed that GBF DNA-binding activity is present in both cell types (Schnitzler *et al.*, 1994). Fusion of the cloned 2.4-kb GBF promoter fragment to the *E. coli lacZ* gene (GBF/*lacZ*) confirmed this result. Expression of GBF/*lacZ* during the slug stage of development is seen throughout the organism (data not shown).

GBF Promoter Activity Does Not Require the Presence of GBF Protein

We undertook several lines of experimentation to determine whether GBF protein is required for the induction of *GBF* expression to wild-type levels. First, we constructed a new *gbf*-null strain that contains a homologous insertion or "Knock-In" of the luciferase gene into the GBF locus (*gbf*/luciferase-KI; Fig. 6A). This strain displays a developmental phenotype identical to those of other *gbf*-null strains and is rescued by constitutive expression of GBFΔI (Fig. 6B; Schnitzler *et al.*, 1994). Comparison of *GBF* mRNA induction in wild-type cells to luciferase mRNA induction in *gbf*/luciferase-KI produced a similar profile in suspension assays (Fig. 6C). Expression of both genes gradually rises over 5 h of starvation and increases substantially upon stimulation with 300 μM cAMP. We did not observe this increase in the absence of cAMP treatment.

Luciferase activity in *gbf*/luciferase-KI cells and *gbf*-null cells transformed with a reporter construct containing the cloned GBF promoter fused to the luciferase gene (*GBF*/luciferase) revealed a similar increase over the first 12 h of development, when both strains undergo mound arrest (Fig. 7D). These data indicate that the cloned and endogenous GBF promoters are regulated similarly. We compared the activity of the *GBF*/luciferase reporter cassette in wild-type

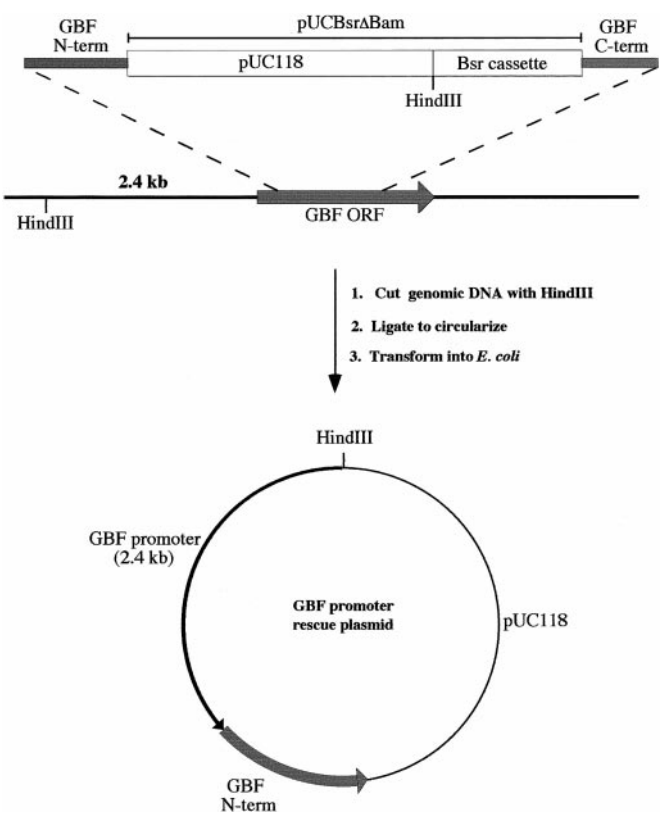

FIG. 5. GBF promoter cloning strategy. pUCBsrΔBam was integrated into the GBF ORF by homologous recombination. A *Hin*dIII site 2.4 kb upstream of the GBF open reading frame was used for plasmid rescue. Construction of the integration cassette is described in Materials and Methods.

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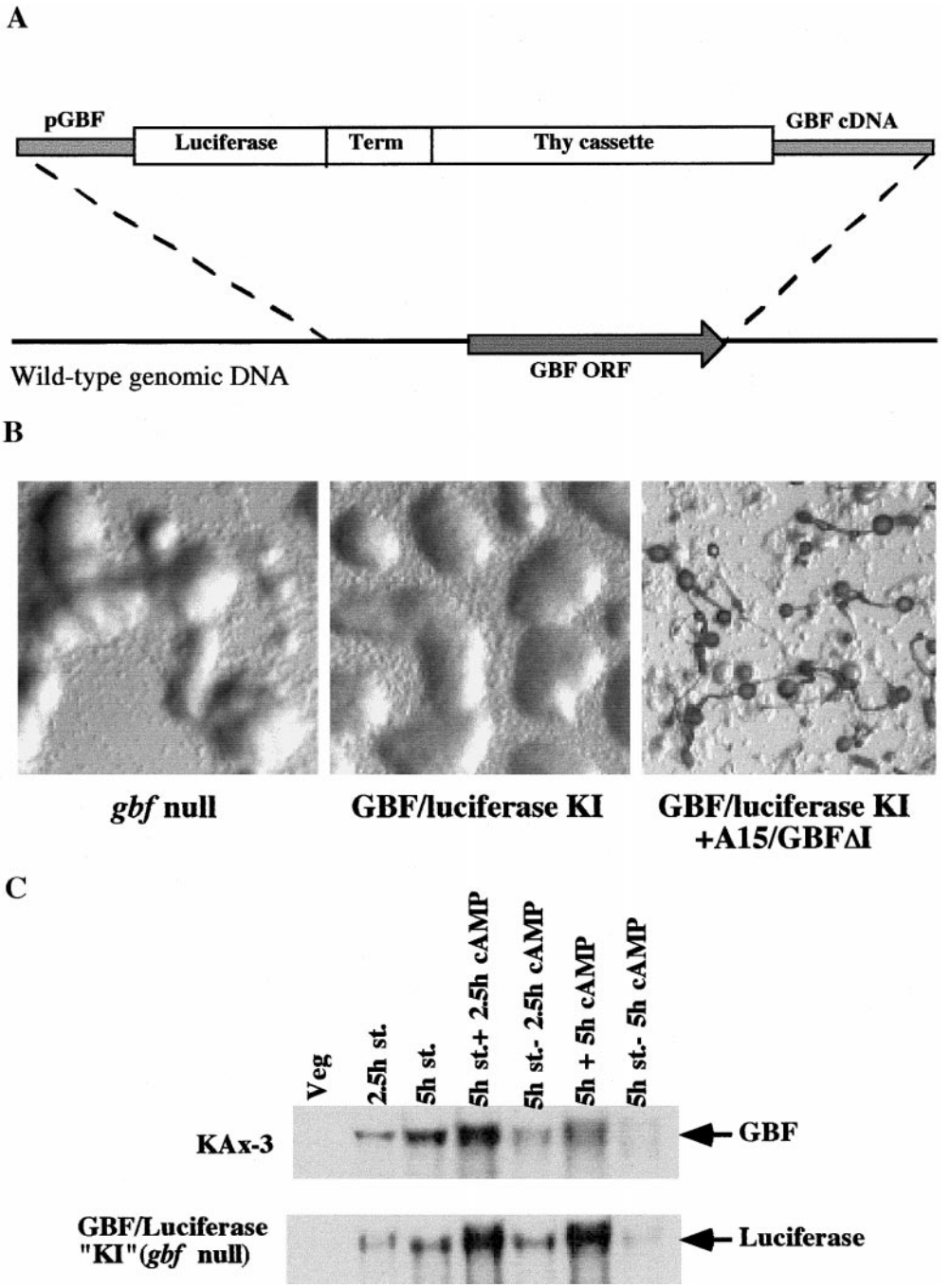

FIG. 6. Construction of GBF/luciferase KI. (A) A cassette containing the luciferase gene, 2H3 terminator, and the Thy selection cassette was integrated into the GBF locus. A 1.1-kb fragment of GBF promoter sequence and a 1.8-kb fragment of GBF cDNA were used for homologous recombination. Construction of the cassette is described in Materials and Methods. (B) Successful integration resulted in the mound-arrest phenotype characteristic of *gbf*-null cells. Constitutive expression of GBFΔI restored the ability to develop fruiting bodies. All pictures are of terminal development. (C) *In vitro* suspension assays were used to examine the cAMP-induced expression of genes at the *GBF* locus. Wild-type *GBF* induction was compared to luciferase in *gbf*/luciferase-KI cells. Cells of each strain were washed, starved for 5 h, and treated (+) or not treated (−) with 300 μM cAMP for 5 h. Total mRNA harvested from each sample was size separated and probed with the appropriate gene.

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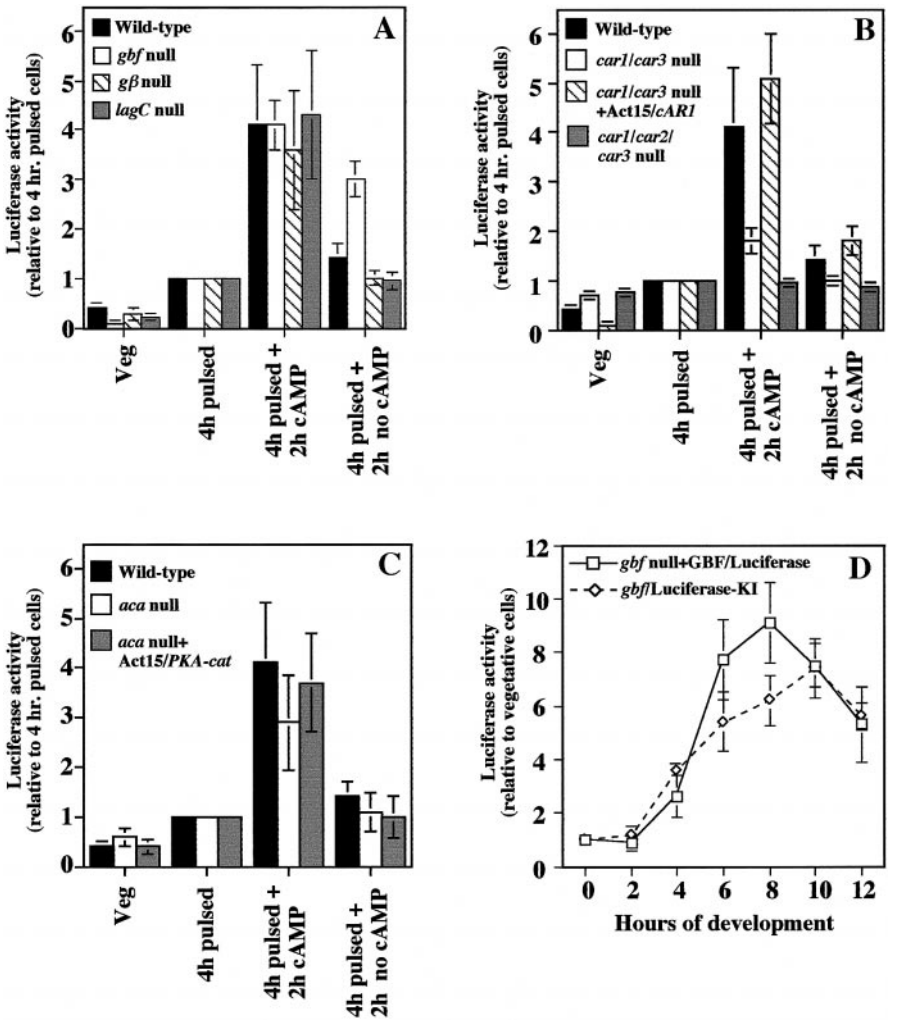

FIG. 7. Activity of GBF/luciferase in various genetic backgrounds. (A–C) Strains transformed with *GBF*/luciferase were pulsed with 30 nM cAMP in suspension for 4 h and starved an additional 2 h with or without 300 μM cAMP. Luciferase activity values are normalized by the total protein concentration of each sample and shown relative to the 4-h pulsed value to emphasize the induction upon treatment with high, continuous cAMP. (D) Cells were developed on phosphate-buffered agar for 12 h, when both strains underwent mound-stage arrest.

and *gbf*-null cells *in vitro* (Fig. 7A). Cells were pulsed for 4 h with 30 nM cAMP to induce the expression of aggregation-stage gene products, followed by stimulation with 300 μM cAMP for 2 h (Insall *et al.*, 1994; Schnitzler *et al.*, 1994). In wild-type cells, expression of *GBF*/luciferase increases ~3-fold in response to pulsing and an additional 4-fold upon stimulation with 300 μM cAMP. In the absence of 300 μM cAMP treatment, we observed little further increase. We found a similar induction of *GBF*/luciferase in *gbf*-null cells, although moderately higher expression occurs in the absence of cAMP (Fig. 7A). Finally, we examined the expression of *lacZ* driven by the GBF promoter in *gbf*-null cells. Although these cells arrest at the mound stage due to the absence of GBF, β−galactosidase activity is clearly detected after 12 h of development (data not shown).

GBF Promoter Activity in Signaling Mutant Strains

Unlike that of other postaggregative genes, such as *LagC* and *CP2* (Aubry and Firtel, 1999), GBF promoter activity in signaling mutant strains does not require GBF protein activity. Therefore, a different regulatory pathway must control its expression. To identify components involved in *GBF* induction, we transformed the *GBF*/luciferase reporter construct into strains containing null mutations in signaling molecules known to be important for *Dictyostelium* development. Expression of *GBF*/luciferase in *car1*/*car3*-null cells (Insall *et al.*, 1994), in which the genes encoding the aggregation-stage cAMP receptors cAR1 and cAR3 have been deleted, is substantially reduced compared to expression in wild-type cells (Fig. 7B). We observed only a slight

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increase after 4 h of pulsing, and addition of 300 μM cAMP resulted in a <2-fold increase. This is likely to be due to a low level of cAR2, another cAMP-receptor isotype expected to be present after several hours of starvation (Saxe III *et al.*, 1993). To address this possibility, we constructed a strain in which *cAR1*, *cAR2*, and *cAR3* are disrupted. Expression of *GBF*/luciferase in this strain is completely absent, in response to pulses or high, continuous concentrations of cAMP (Fig. 7B). Constitutive expression of cAR1 in *car1*/*car3*-null cells fully restores the ability to express *GBF*/luciferase at wild-type levels (Fig. 7B). Our results strongly suggest that the cAMP-induced increase in *GBF* expression is mediated by cell-surface receptors.

We measured *GBF*/luciferase activity in *gβ*-null cells, which are deficient in the only known *Dictyostelium* Gβ subunit (Fig. 7A; Lilly *et al.*, 1988; Wu *et al.*, 1995). cAMP-induced expression in this strain is virtually identical to that seen in wild-type cells, indicating that, like the cAMP-mediated stimulation of GBF protein activity, induction of GBF expression is G-protein-independent. *LagC*, which encodes a cell-surface molecule required for continuous, high-level expression of GBF throughout later development (Dynes *et al.*, 1994; Sukumaran *et al.*, 1998), is also not required for the initial induction of GBF (Fig. 7A).

Finally, we examined the role of cAMP-dependent protein kinase (PKA) in GBF induction. PKA is required for numerous aspects of *Dictyostelium* development, including aggregation, prespore gene expression, and sporulation (reviewed in Aubry and Firtel, 1999; Loomis, 1998). Previous results indicated that both GBF DNA-binding activity and GBF-mediated gene expression are functional in cells containing a deletion of the PKA catalytic subunit gene (*pka-cat* null; Mann *et al.*, 1997). To further investigate the role of intracellular cAMP in *GBF* expression, we studied *aca*-null cells, which do not contain the primary aggregation-stage adenylyl cyclase ACA. We observed no significant difference in *GBF*/luciferase activity in this strain (Fig. 7C). Constitutive expression of PKA-cat in *aca*-null cells does not significantly affect GBF promoter activity compared to *aca*-null cells and does not alter *GBF*/luciferase expression in the absence of cAMP stimulation (Fig. 7C), providing further evidence that PKA does not play an important role in GBF induction.

DISCUSSION

Mutational Analysis of GBF

As *Dictyostelium* cells complete aggregation, increasing cAMP concentrations stimulate the activity of GBF, a transcription factor required for high-level expression of genes involved in postaggregative development. GBF does not confer spatial or temporal specificity, but likely acts in concert with other factors that respond to localized signals and contribute this information. We have analyzed the function and transcriptional regulation of GBF to facilitate our understanding of the developmental transition from aggregation to morphogenesis.

Mutational analysis has identified several functional and nonessential domains within the GBF protein. Deletion of amino acids 10–77 or 532–708 does not affect the ability of GBF to complement the *gbf*-null phenotype or bind a G-box-containing oligonucleotide *in vitro*. However, deletion of additional residues (residues 77–115 or 400–532) creates a hypomorphic allele. *gbf*-null cells expressing these alleles form tight mounds with elongated tips and are able to induce the expression of *LagC*, but not *CP2*, in response to cAMP in a cell suspension assay. The results suggest that different domains of the GBF protein may be required for the transcriptional activation of subsets of target genes. The deleted portions of the protein are most likely required for physical interaction with other transcriptional activators or coactivators specific to the misregulated genes. Alternatively, the *in vivo* binding affinity of the mutant proteins may be altered to various extents, which could differentially affect the activation of GBF-dependent genes. Results from additional studies indicated that deletion of the region between amino acids 283 and 338 causes an inability to progress past the mound arrest stage observed in *gbf*-null cells. This mutant exhibits sequence-specific DNA binding *in vitro*, suggesting that the deleted domain is required for transcriptional activation. Examination of GBF mutants containing deletions of both N- and C-terminal sequences revealed some functional overlap. Residues 59–77 were only required for fruiting body formation in the absence of amino acids 532–708. There is no direct sequence conservation between these two parts of the protein, although both contain a high proportion of serine and threonine residues.

As expected, point mutations in conserved cysteine residues predicted to be required for folding of the zinc finger domains or deletion of the C-terminal zinc finger cause a loss of DNA binding *in vitro*. It was surprising, however, to find that, when the zinc finger mutants are expressed in *gbf*-null cells, the mound arrest phenotype is complemented, indicating that these proteins retain *in vivo* function. Although we cannot exclude the possibility that the zinc finger mutants function independent of DNA binding, we consider this unlikely, given the evidence supporting the model that direct binding of wild-type GBF to promoter elements is required for the expression of cAMP-stimulated postaggregative genes. Deletions or point mutations in G-boxes in the *CP1*, *CP2*, and *SP60/cotC* promoters that disrupt GBF binding *in vitro* cause a substantial reduction in gene expression *in vivo* (Ceccarelli *et al.*, 1991; Datta and Firtel, 1987; Datta *et al.*, 1987; Esch *et al.*, 1992; Fosnaugh and Loomis, 1993; Haberstroh and Firtel, 1990; Haberstroh *et al.*, 1991; Pears and Williams, 1987; Powell-Coffman and Firtel, 1994). In addition, *gbf*-null cells do not express these genes, either during development or in response to cAMP in suspension cultures (Schnitzler *et al.*, 1994, 1995). Finally, GBF protein synthesized *in vitro* causes the same size mobility shift in an EMSA as the GBF activity present in

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cellular extracts, favoring the notion that GBF binds DNA directly and not via accessory proteins (Schnitzler *et al.*, 1994). Although in both of these cases the basal transcription apparatus could participate in DNA binding, this would not account for the observed sequence specificity.

We expect that the disruption of one zinc finger causes a reduction in the affinity of GBF for G-boxes that prevents DNA binding *in vitro*, but potential cooperative interactions with locus-specific factors may stabilize DNA binding activity *in vivo*, allowing the induction of GBF-regulated genes. The 43-bp portion of the CP2 promoter used in our gel shift assays contains only an isolated GBF binding site (Hjorth *et al.*, 1989). Such isolated GBF response elements do not direct transcription *in vivo* and presumably function only in context with other required *cis* elements (Pears and Williams, 1988). Our finding that some GBF mutants are only able to activate subsets of target genes supports this hypothesis. The regulatory activity of the mammalian erythropoeic factor GATA-1 is modulated in such a manner (Mackay and Crossley, 1998). DNA binding occurs via the C-terminal zinc finger of GATA-1, whereas the N-terminal zinc finger is primarily utilized for a variety of protein–protein interactions that influence the regulatory activity of GATA-1 (Crossley *et al.*, 1995; Fox *et al.*, 1999; Mackay and Crossley, 1998; Osada *et al.*, 1995; Tsang *et al.*, 1997; Visvader *et al.*, 1995). We expect that similar protein–protein interactions are important for the ability of GBF to activate postaggregative and cell-type-specific genes with the correct spatial and temporal specificity. However, our data suggest that these interactions are more likely to depend on domains other than the zinc fingers of GBF. Finally, although mutational analyses of GBF-regulated promoters indicate that two intact CA/GT-rich half-sites are required for GBF binding and transcriptional activation, we have found no evidence of dimer formation in cells coexpressing wild-type GBF and an N- or C-terminal deletion mutant. This result supports the model in which, *in vivo*, a single molecule of wild-type GBF binds to a G-box, with each zinc finger interacting with a half-site. As discussed above, stabilizing interactions with other factors likely allow a similar binding topology for GBF zinc-finger mutants. However, we cannot exclude the possibility that disruption of one zinc finger alters the stoichiometry of GBF binding to the G-box, as these mutants were not detectable by EMSA.

Regulation of GBF Expression

The increase in *GBF* transcription in response to rising cAMP concentrations in mounds is a key step in postaggregative development. Our finding that *GBF* promoter-directed transcription does not require the presence of GBF protein demonstrates that this increase is not due to a *GBF*-mediated auto-induction loop, as previously hypothesized. We attempted to identify components of the *GBF* induction pathway by assaying the activity of *GBF* promoter/luciferase fusions in a variety of mutant backgrounds. As expected, the cAMP-stimulated *GBF* expression is absent in *cAR1/cAR2/cAR3*-null cells and fully restored by constitutive expression of cAR1 in *cAR1/cAR3*-null cells. These observations indicate that *GBF* induction is receptor-dependent, although we cannot rule out the possibility that this effect is indirect, owing to the absence of one or more pulse-induced factors. In either case, the cAMP-stimulated signaling pathway that induces *GBF* expression must be at least partially distinct from that which causes GBF protein activation; there is a branch somewhere downstream of the cAMP receptor.

As in previous studies aimed at finding signaling components essential for GBF protein activation, other key regulators are elusive. *GBF*/luciferase activity in *gβ*-null cells is comparable to that observed in wild-type cells, ruling out involvement of G-proteins in this pathway. This adds to the growing list of cAMP-receptor-dependent responses in *Dictyostelium* that do not require G-proteins, including the stimulation of GBF activity (Schnitzler *et al.*, 1995), the activation of MAP kinase (ERK2) activity (Maeda *et al.*, 1996), the phosphorylation, dimerization, and nuclear localization of DdSTATa (Araki *et al.*, 1998), and cAMP-stimulated Ca^{2+} influx (Milne *et al.*, 1995). The universal importance of G-protein-independent signaling events regulated by serpentine receptor stimulation has been highlighted by the recent description of such pathways in higher eukaryotes, including mammalian cells (reviewed in Hall *et al.*, 1999). Instead of coupling to G-proteins, the serpentine receptors that control these pathways signal via other types of signaling molecules, such as small G-proteins (Mitchell *et al.*, 1998), SH2-domain-containing proteins (Karoor *et al.*, 1998; Marrero *et al.*, 1998; Venema *et al.*, 1998) and G-protein-coupled receptor kinases (Carman *et al.*, 1998; Haga *et al.*, 1998; Luttrell *et al.*, 1999; Pitcher *et al.*, 1998; Premont *et al.*, 1998). At least one member of each of these classes of proteins is essential for proper *Dictyostelium* multicellular development (Mohanty *et al.*, 1999; Tuxworth *et al.*, 1997; Briscoe *et al.*, 2001) and may play prominent roles in these newly identified pathways.

Preliminary mutational analysis of the GBF promoter indicates ~700–800 bp upstream of the GBF start codon are essential for expression *in vitro*. This region contains two CA-rich half-sites that conform to the G-box consensus, suggesting that another unknown G-box factor(s) may recognize these *cis* elements. If this is so, this factor(s) cannot replace GBF in the context of most postaggregative and cell-type-specific promoters. It is also possible that these are sites of negative regulation by GBF or that they are nonfunctional and GBF expression is mediated by as-yet-unrecognized *cis* elements. Further GBF-promoter mutational analyses may reveal the nature of GBF transcriptional regulation.

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