Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in *Dictyostelium*

Gavin R. Schnitzler,¹ Wolfgang H. Fischer,² and Richard A. Firtel^{1,3}

¹Department of Biology, Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0634 USA; ²Clayton Foundation Laboratory for Peptide Biology, The Salk Institute for Biological Sciences, La Jolla, California 92037 USA

During *Dictyostelium* development, the cAMP-regulated induction of cell-type-specific late genes marks a developmental switch from the initial formation of the multicellular organism to the differentiation of the various cell types that mediate morphogenesis and eventually give rise to the mature fruiting body. The G-box binding factor (GBF) is a developmentally regulated *Dictyostelium* transcription factor whose affinity for a DNA sequence correlates with the ability of that sequence to confer inducibility to late gene promoters in response to high, continuous levels of extracellular cAMP. We report the purification of GBF and cloning of the gene that encodes it, as confirmed by in vitro production of GBF activity. The predicted protein is highly basic and contains two putative zinc fingers. Disruption of the GBF gene by homologous recombination results in the loss of all GBF DNA-binding activity, developmental arrest at the loose aggregate stage, and the loss of late gene induction during development or in response to extracellular cAMP. Constitutive expression of GBF complements the null phenotype and allows for the rapid activation of a class of late genes in response to cAMP. Our results indicate that GBF acts as an extracellular cAMP-responsive transcriptional activator regulating late gene expression and is an essential component of a developmental switch between aggregation and cellular morphogenesis.

[Key Words: *Dictyostelium discoideum*; transcription factor; DNA-binding protein; signal transduction]

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An early aggregate of *Dictyostelium discoideum* cells, formed by the chemotactic aggregation of individual amoebae, must undergo a critical transition whereby it becomes a coherent multicellular organism composed of a number of distinct cell types. High levels of cAMP and other signals in the aggregate mediate this transition by the activation of a variety of genes involved in later development and cellular morphogenesis (for review, see Kimmel and Firtel 1991; Williams 1991). These late genes display a variety of temporal and spatial expression patterns. Some, such as *pst-cathepsin/CP2* (hereinafter called *CP2*) and *DdrasD* are activated during aggregation and require only high, continuous levels of extracellular cAMP and starvation signals for their expression (Mehdy et al. 1983; Mehdy and Firtel 1985; Raymond et al. 1984). We will refer to these genes as primary late genes. Others, referred to here as secondary late genes, are activated later and require additional signals that may be present only at tight-aggregate or tipped-aggregate stages. For ex-

ample, *SP60* and *SP70* require as-yet-unspecified cell-cell contacts (Mehdy et al. 1983; Mehdy and Firtel 1985; Fosnaugh and Loomis 1991), and *ecmA* and *ecmB* require the morphogen DIF (Williams et al. 1987; Berks and Kay 1990). For some, expression is restricted to prestalk and anterior-like cells, such as *ecmA* and *ecmB* (Jermyn and Williams 1991), or is prestalk enriched, such as *CP2* (Pears et al. 1985; Gomer et al. 1986a) and *DdrasD* (Raymond et al. 1984; Jermyn et al. 1987; Esch and Firtel 1991). Others, such as *SP60* (Haberstroh and Firtel 1990), *PspB* (Powell-Coffman and Firtel 1994), and *SP70* (Fosnaugh and Loomis 1993), are expressed only in prespore cells. What unites these diverse genes is their late activation (after the onset of aggregation) and their induction by, or requirement for, continuous micromolar levels of cAMP (Mehdy et al. 1983; Mehdy and Firtel 1985; Haribabu and Dottin 1986; Berks and Kay 1990; Fosnaugh and Loomis 1991).

The promoters of many late genes contain CA/GT-rich sequences that have been shown to be essential for their activation during development or in response to cAMP [Haberstroh and Firtel 1990; Haberstroh et al.

³Corresponding author.

1991 (*SP60*); Fosnaugh and Loomis 1993 (*SP70*); Powell-Coffman and Firtel 1994 (*PspB*); Ceccarelli et al. 1991, 1992 (*ecmB*); Early et al. 1993 (*ecmA*); Datta and Firtel 1987, 1988; Pears and Williams 1987 (*pst-cathepsin/CP2*); Pavlovic et al. 1989 (*UDPGP*); Esch et al. 1992 (*DdrasD*)). The G-box binding factor, or GBF, is a developmentally regulated activity that binds specifically to a number of CA/GT-rich late gene regulatory elements [Hjorth et al. 1989 (*CP2* and *DdrasD*); Hjorth et al. 1990 (*UDPGP*); Ceccarelli et al. 1992 (*ecmB*); J. Powell-Coffman, G. Schnitzler, and R.A. Firtel, in prep. (*SP60*); Powell-Coffman and Firtel 1994 (*PspB*)], as well as similar sequences from late gene promoters yet to be analyzed for *cis* elements [Hjorth et al. 1990 (*CP1* and *DG17*)]. Each of these GBF-binding sequences contains two half-sites of the rough consensus (T/G)G(G/T)G(T/G)G(T/G), and the spacing and orientation between these sites is flexible [Hjorth et al. 1990; G. Schnitzler and R. Firtel, unpubl.]. Where it has been tested, GBF's affinity for a binding site in vitro correlates with the ability of that sequence to restore cAMP inducibility to a mutated late gene promoter, *CP2Δ30*, in vivo [Pears and Williams 1988; Hjorth et al. 1989, 1990; C. Gaskins, G. Schnitzler and R. Firtel, unpubl.]. In addition, mutant sequences that act as poor GBF binding sites in vitro function poorly in facilitating the developmental expression of the *SP60* promoter [Haberstroh et al. 1991; J. Powell-Coffman, G.R. Schnitzler, and R.A. Firtel, in prep.]. Taken together, these data indicate that GBF may be a transcriptional activator of a wide variety of late genes in response to cAMP.

To examine further the role of this factor in the regulation of late genes, we have purified GBF and cloned the gene that encodes it. This is the first DNA binding protein to be cloned by virtue of its function in *Dictyostelium*. Analysis of *gbf*⁻ null cells demonstrates that GBF is required for post-aggregative development and late gene expression in response to extracellular cAMP. Constitutive expression of GBF allows cells to rapidly activate the primary late gene *CP2* in response to cAMP. Our results indicate that GBF functions as a transcription factor that is part of a developmental switch between the initial formation of the multicellular organism and cellular morphogenesis.

Results

Purification and apparent molecular weight of GBF

Earlier examination of GBF DNA-binding activity used nuclear extracts because of difficulties in observing specific complexes in cytosolic extract preparations [Hjorth et al. 1989]. Optimization of the mobility-shift assay (see Materials and methods) has made it possible to observe a specific GBF complex (as determined by Rf comparison and competition with wild-type and mutant GBF-binding sites) in cytosolic extracts. GBF activity is ~22-fold enriched in the cytosol (Fig. 1A), and present as ~5000 molecules per 17-hr developed cell.

GBF was purified ~3000-fold from 17-hr cytoplasmic

Figure 1. GBF purification and apparent molecular mass. (A) Cytosolic and nuclear extracts were prepared from 17-hr developed cells and tested in an electrophoretic mobility shift assay (EMSA), as described in Materials and methods, with 800 ng of poly[d(I-C)] as a nonspecific competitor. Specific competitors are CP2, mutant CP2 (CP2m), and CAE1 as indicated. (B) DEAE fractions were precipitated with TCA and subjected to SDS-PAGE on a 7.5% acrylamide gel. Molecular weight markers are from Bio-Rad, and the gel was stained with silver nitrate. (C) The 92-kD GBF band in the DEAE flowthrough fraction was excised from a Coomassie blue stained 7.5% SDS-PAGE gel, and proteins were eluted, renatured, and tested by EMSA using only 200 ng poly[d(I-C)]. CP2 competitor is present in the indicated lanes. A CAE1 affinity column control fraction is also shown exposed for ~10-fold less time.

extracts, with a 40% recovery of binding activity, by use of sequential chromatography on DNA cellulose, *SP60* CAE-1 affinity resin and DEAE Sepharose (Table 1). To use SDS-PAGE as a final stage in the purification process, it was necessary to determine the apparent molecular mass of all protein species that constitute GBF activity. To accomplish this, various fractions from the

Table 1. Purification of GBF activity

Fraction	mg	Units GBF	Fold purification
17-hr cytosolic extract	751	285,500	—
DNA-cellulose	12	174,000	38
CAE1 affinity	~1.8	146,000	~215
DEAE FT	~0.1	115,100	~3000

Results of the purification of cytosolic extracts from 1×10^{11} 17-hr developed cells are shown. Protein levels were determined by Bio-Rad protein reagent for extracts and DNA-cellulose fractions, and estimated by examination of stained SDS-PAGE gels for later fractions. Units of GBF activity were calculated as described in Materials and methods.

purification were size-separated by SDS-PAGE, lanes were cut into sections, and proteins eluted, renatured, and assayed (see Materials and methods). By use of crude extracts, specific GBF activity was demonstrated to run between 68 and 95 kD, and renatured proteins from other size ranges could not increase or modify this binding activity (data not shown). In fractions from the DEAE chromatography, a single band at ~92 kD (comprising ~30% of total protein; see Fig. 1B) was isolated and renatured to give specific GBF activity (Fig. 1C). The amount of this band correlated with level of GBF activity throughout the purification (data not shown). From these results, we concluded that GBF activity can be accounted for by a single 92-kD protein species (or multiple species of the same size).

Isolation of tryptic fragments and cloning of the gene encoding GBF

DEAE fractions containing GBF activity were precipitated, run on SDS-PAGE, blotted onto PVDF membrane, and stained (see Materials and methods). The isolated 92-kD band was subjected to digestion with trypsin, and peptides released were isolated by HPLC. Four HPLC peaks were sequenced yielding the following partial peptide sequences: 1033, (S)NI(T)PI(T)D(S)(L/S)(T)(S)(D); 1035, (L/K)PFTP(W/V)G AG(L/K)SP(S); 1037, AFTPGITK; 1042, DS(S/C)GQTIPK. Letters in parentheses indicate indefinite assignments, with letters preceding a slash indicating the most likely amino acid.

Six degenerate oligonucleotides were designed from three of the peptide sequences (1035, 1037, and 1042), as sense and antisense pairs, and used in pairwise combinations to amplify *Dictyostelium* genomic DNA by PCR. One combination (1037-sense and 1035-antisense) gave a product of ~480 bp, which was cloned and used to isolate multiple, overlapping, partial cDNAs from a 12-

to 16-hr developmental cDNA library (Fig. 2). A Southern blot using the PCR product as probe indicated that the GBF gene is present in a single copy (data not shown). A genomic map of the region surrounding GBF is presented in Figure 2. The PCR product was used to screen a *Dictyostelium* YAC library (Kuspa et al. 1992), and it hybridized to a single YAC, which has not yet been incorporated into a linkage group (A. Kuspa and W.F. Loomis, pers. comm.).

GBF protein contains two putative zinc fingers

The overlapping cDNAs contain one long open reading frame (starting at position 215 and ending at position 2340 from the 5' end of the cDNAs). This encodes a 708-amino-acid protein (predicted molecular mass of 79.3 kD), in which all four of the sequenced peptides could be found (underlined sequences, Fig. 3A), confirming that the gene cloned encodes the protein that was purified. The protein contains very few acidic residues and has a predicted pI of 9.76. The ~13-kD difference between observed and predicted molecular mass might result in part from the basic nature of the protein, as highly basic proteins, such as histone H1, often run on SDS-PAGE with an apparent molecular mass larger than their actual mass (Lennox and Cohen 1989), or may indicate that GBF is post-translationally modified in vivo. GBF is predicted to be highly hydrophilic (Kyte and Doolittle 1982) and to contain a preponderance of turns, occasional short stretches of β -sheet and very little α -helix (Chou and Fasman 1978; Garnier et al. 1978; data not shown).

GBF has no extensive homology to any known protein (BLAST search of nr library at NCBI; Altschul et al. 1990), except over the long Gln- and Asn-rich stretches of GBF. One such region of GBF (63% Gln and 19% His over 150 amino acids) contains four near-perfect direct

Figure 2. GBF genomic organization and constructs. The genomic organization of GBF was determined by restriction endonuclease digestion and Southern blotting with the subcloned PCR product and GBF cDNAs as probes. The GBF cDNA, as well as transformation and expression constructs, are shown aligned with the genomic DNA (see Materials and methods for details). White and black boxes indicate coding and noncoding GBF sequences, respectively. Dark grey boxes within the GBF coding region show the positions of the two Cys₄ repeats. Comparison of endonuclease digestions of the cDNA and genomic DNA suggests the absence of long introns within the region encompassed by the cDNA. In the constructs, black lines indicate vector sequences, and labeled, shaded boxes are non-GBF *Dictyostelium* and/or bacterial sequences. Arrows indicate the direction and position of promoters used in the expression constructs. Thy1 represents the *Thy1* gene used for auxotrophic selection in JH10 cells for the gene disruption (see Materials and methods). Restriction enzyme sites shown are *Bgl*III (B), *Xba*I (X), *Spe*I (S), *Nde*I (N), *Hind*III (H), *Kpn*I (K), *Cla*I (C), *Eco*RI (R), *Hinc*II (I), *Apa*I (A), and *Sac*I (Sa).

C

IPKCTRNEAA^{*}SWKHKD^{*}RR^{*}WWCKECKKAFTPG
CPPCPLCRGIS^{*}SWKHKDKRY^{*}YFCKECKKPFETPV
CRSCHYHRRNTGDPDIM-^{*}CSLCYMRTC
CLVCSDEA^{*}SGCHYGVLT-^{*}CGSCKVFFK
KQFICKYCNQFT
EKPYKCEECGKAFT

GBF 5' C4 repeat
GBF 3' C4 repeat
Aden. E1A finger
GR 5' Zn twist
Odd-skipped 5' of 1st C₂H₂
Zn43 (human)

505

Figure 4. Electrophoretic mobility shifts of in vitro-translated GBF and separated cell extracts. (A) One microliter of DNA-cellulose-purified GBF is compared with 1 μ l of TnT rabbit reticulocyte lysates programmed with the GBF expression construct R20 (Txn/Txl) or a control construct expressing luciferase (Luc.) by EMSA. The dried gel was exposed to X-ray film with an intervening film to screen out 35 S radiation from labeled proteins. (B) KAx-3 cells were developed for 16 hr at a density of 5×10^6 cells/cm 2 on buffered agar and separated into prestalk and prespore populations by Percoll gradient centrifugation (see Materials and methods). Cytoplasmic extract of separated cells (1.2 μ g) was examined by EMSA. CAE1 competitor is present in the indicated lanes. Fold enrichment was determined by use of *ecmA* (prestalk-specific) and *PspB* (prespore-specific) RNAs as markers to measure cross-contamination of the separated cell types.

throughout the remainder of development (see Fig. 7, below). The transcript is 1.2 kb larger than the contiguous cDNAs isolated so far, which is presumably due to additional 5'- and/or 3'-untranslated sequence. Other RNA time courses show a low level of GBF transcripts at 3 and 4 hr (data not shown). The variability at the 4-hr time point may indicate that GBF expression is first induced at this time, making it sensitive to slight differences in developmental conditions. The RNA time course correlates with the developmental induction of GBF-binding activity in extracts, which is low in vegetative cells and at 5 hr but increases to a steady-state level at 10 hr (Hjorth et al. 1989; see Discussion).

All late genes tested can be induced in shaking culture with the addition of micromolar levels of extracellular cAMP after a period of starvation. The primary late genes (such as *CP2* and *DdrasD*, see introductory section) can be activated under fast-shake conditions, in which high rotational speed and EDTA keep cells from clumping (Mehdy et al. 1983; Mehdy and Firtel 1985; Reymond et al. 1984). Others, such as *SP60* (Mehdy et al. 1983; Mehdy and Firtel 1985; Fosnaugh and Loomis 1991) and *ecmA* (see Fig. 8, below; Berks and Kay 1990), require the

clustering of cells allowed in slow-shake cultures. GBF activity has been shown to be induced by starvation followed by cAMP in slow-shake culture (Hjorth et al. 1989). Figure 8 (below) shows that the GBF transcript can be activated fivefold by starvation for 6 hr. This expression level is the same at 3 and 9 hr of starvation (data not shown), and a similar increase in GBF-binding activity is observed in extracts from 4-hr starved cells (see Fig. 6A, below). Unlike early aggregation-stage genes, *GBF* expression is largely unaffected by 30 nM pulses of cAMP given every 6 min for either 3 or 6 hr (data not shown). *GBF* expression is, however, rapidly activated by 300 μ M cAMP under both fast- and slow-shake conditions (three- to fourfold in 1 hr). GBF transcription is activated in slow shake without cAMP, an effect also observed for other primary late genes [Mehdy et al. 1983 (*CP2*); Reymond et al. 1984 (*DdrasD*); see below], which may result from a buildup of cAMP, or other potential activating signals, in clumps of cells.

To examine the cell-type distribution of GBF, RNAs from 16-hr developed cells were separated by Percoll gradient centrifugation into prespore and prestalk populations (see Materials and methods). GBF was present in cytoplasmic extracts made from both prestalk and prespore cells, although it was 2.8-fold enriched in prespore cells (Fig. 4B). GBF transcripts showed a similar (2.5-fold) enrichment in prespore cells (data not shown).

GBF gene disruptants are arrested at the loose mound stage

A GBF gene disruption was made by use of the *Thy1* selectable marker transformed into JH10 cells (Fig. 2). Whereas all nondisrupted clones displayed wild-type development, all disrupted clones (*gbf* $^{-}$) were delayed in aggregation by ~ 2 hr and arrested at the loose aggregate stage (Fig. 5A), forming high mounds after ~ 12 hr, but never producing a tip or sheath. At ~ 20 hr, the high mounds partially disperse, only to reform ~ 4 –6 hr later (cf. *gbf* $^{-}$ null cells at 12, 20, and 24 hr; Fig. 5A).

To address whether *gbf* $^{-}$ null cells could participate in development along with wild-type cells, *gbf* $^{-}$ cells were transformed with a construct containing the β -galactosidase gene driven by the *Actin15* (*Act15*) promoter. This promoter is expressed most strongly in early development yet continues to be expressed at reasonable levels throughout development (Knecht et al. 1986) and roughly equally in all cell types (Mann and Firtel 1993). These cells were then mixed with wild-type cells, allowed to develop for various times, fixed, and stained for β -galactosidase (see Materials and methods). Figure 5B shows that *gbf* $^{-}$ cells can move to the same aggregation centers as wild-type cells, although perhaps more slowly, but are then left behind when the wild-type cells form later developmental structures, demonstrating the cell autonomous nature of GBF function.

Cytoplasmic extracts of vegetatively grown *gbf* $^{-}$ null cells and extracts of *gbf* $^{-}$ cells after 4 hr of starvation in shaking culture or 16 hr of development showed no detectable GBF-binding activity (Fig. 6A; data not shown).

Figure 5. Morphological phenotypes of *GBF* disruptants and overexpressors. (A) Cells were grown axenically, washed, and plated at between $\sim 7.5 \times 10^5$ and $\sim 1.5 \times 10^6$ cells/ml on sodium phosphate-buffered agar. Cell line and time of development are indicated. (B) Wild-type KAx-3 and a clonal population of *gbf*⁻ cells transformed with the *Act15-lacZ* construct were grown axenically, washed, mixed in a 1:1 ratio, and plated at $\sim 1 \times 10^6$ cells/ml on Millipore filters over buffer. At the indicated times in development (8 or 16 hr) the cells were fixed and stained with X-gal as described in Materials and methods.

This result confirms that the cloned *GBF* gene is responsible for all of the detectable *GBF*-binding activity in extracts. In addition, *gbf*⁻ cells produced no *GBF* transcript under either shaking culture or plated development conditions (Figs. 8 and 7, respectively).

Constitutive GBF expression complements gbf⁻ cells and accelerates development

To prove that the disruption phenotype is not due to a secondary mutation, the complete *GBF* cDNA was in-

roduced into *gbf*⁻ null cells under control of the *Act15* promoter (construct A15-*GBF*). All A15-*GBF/gbf*⁻ clones contained multiple tandem copies of the introduced DNA and expressed high levels of a 3.6-kb A15-*GBF* transcript (data not shown). Each transformant displayed at least a partial rescue of the *gbf*⁻ phenotype (Fig. 5A). The somewhat abnormal morphology of post-aggregative structures is probably due to differences in the expression pattern of the *Act15* and *GBF* promoters. In cytoplasmic extracts from 4-hr starved A15-*GBF/gbf*⁻ cells, a specific mobility-shift band at the same Rf

Figure 6. GBF activity in extracts of *gbf*⁻, A15, GBF/*gbf*⁻ and A15-GBF cells. (A) Cytoplasmic extracts were prepared from 16-hr developed KAX-3, vegetative JH10 cells, and AX3, *gbf*⁻, and A15-GBF/*gbf*⁻ cells starved for 4 hr in shaking culture. Each extract (1.8 μ g) was tested by EMSA. (B) A cytoplasmic extract of vegetative JH10 and A15-GBF cells (1.8 μ g) was tested by EMSA.

of wild-type GBF was seen (Fig. 6A), further confirming that the cloned sequences encode the complete GBF protein.

The A15-GBF construct was transformed into wild-type KAX-3 cells to generate the A15-GBF cell line. These cells express both the 3.8-kb endogenous GBF and 3.6-kb A15-GBF transcripts (Fig. 7). These overexpressing cells are consistently smaller than wild-type cells when grown in monolayer or shaking culture (data not shown). When plated for development, these cells formed tight aggregates and slugs 1–2 hr ahead of wild-type cells. The morphology of developmental structures is nearly wild type, although slugs are more elongated, and a larger proportion of cells failed to enter aggregates (cf. wild-type cells at 6 and 13 hr to A15-GBF cells at 6 and 13 hr, Fig. 5A). Vegetative cytoplasmic extracts from this strain contain at least eightfold more GBF activity than extracts from wild-type cells (Fig. 6B).

GBF is required for late gene activation by cAMP

We examined the expression of a number of developmental marker gene mRNAs in wild-type, *gbf*⁻ and A15-GBF cells. Most of these genes are late genes that contain important CA/GT rich elements to which GBF has been shown or might be expected to bind (*SP60*, *ecmA*, *CP2*, and *DdrasD*; see introductory section). Two others are

early, aggregation-stage genes that are activated by nanomolar pulses of cAMP and repressed by the continuous micromolar concentrations that activate late genes [Mann and Firtel 1987 (*D2*); Gerisch et al. 1975; Ma and Siu 1990 (*csA*)], and should not require GBF for their expression. *CP2* (Fig. 7) is one of the first late genes to be expressed, beginning at the initial stages of mound formation (~6 hr). In *gbf*⁻ cells the *CP2* transcript is only very weakly expressed at 8 and 20 hr (partially dispersed aggregates) but not at times when high mounds are present. The reinduction at 20 hr is also seen for the early, aggregation-stage genes *D2* and *csA* (see below) and indicates that this residual *CP2* expression is responding to signals that activate these genes. Accordingly, we have found that *CP2* can be activated to a similar, low level by 30 nM pulses of cAMP (A. Clark, G.R. Schnitzler and R.A. Firtel, unpub.), which induce the aggregation-stage genes. Characteristic of primary late genes, *CP2* expression can be highly activated, after a period of starvation, by the addition of continuous micromolar levels of cAMP to fast-shaking culture. This response is abolished in *gbf*⁻ null cells (Fig. 8). Similar

Figure 7. Developmental regulation of GBF and marker genes in wild-type, *gbf*⁻, and A15-GBF cells. RNA was collected from KAX-3 (WILD-TYPE), A15-GBF, and *gbf*⁻ null cells developed at a density of $\sim 6 \times 10^6$ cells/cm² on filters for the indicated times. These RNAs (6 μ g) were size separated, blotted, and probed. Probes are *GBF*, *CP2*, *SP60*, *ecmA*, *csA*, and *D2* as indicated.

Figure 8. Fast- and slow-shake regulation of GBF and developmental marker RNAs in wild-type, *gbf*^{-/-} and A15-GBF cells. KAX-3, *gbf*^{-/-} and A15-GBF cells were grown axenically, washed, and resuspended at 5×10^6 cells/ml. Starvation – cells were either collected or treated with cAMP (with shaking at 250 rpm) immediately after resuspension. Starvation + cells were shaken for 6 hr at 250 rpm before collection or further treatment. Where indicated, cAMP was added to 300 μ M, and cells were collected at the indicated times thereafter. For 6-hr cAMP incubations additional cAMP was added at 2 and 4 hr, to 150 μ M. For fast-shake experiments (F), 2 mM EDTA (pH 6.2) was added at 6 hr, and incubation continued at 250 rpm. For slow-shake

(S), incubation after 6 hr was at 130 rpm. Size-separated RNAs (7 μ g) were blotted and probed with *GBF*, *CP2*, *ecmA*, and *SP60* DNAs. Solid arrows indicate the positions of these transcripts: open arrows indicate weak hybridization to the 3.9- and 1.9-kb ribosomal transcripts. An asterisk in the *gbf*^{-/-} panel probed with *ecmA* marks the position of a spurious band that runs slightly larger than the major *ecmA* transcript.

results were observed for another primary late gene *DdrasD* whose transcripts never accumulate above the low levels present at 4 hr of development or after 6 hr of starvation in *gbf*^{-/-} cells (data not shown). Thus, although primary late genes can respond weakly to early developmental signals in the absence of GBF, their response to continuous micromolar cAMP is completely dependent on GBF.

In A15-GBF cells, the developmental expression of *CP2* is accelerated, first appearing very weakly in vegetative cells, and accumulating to easily detectable levels at 4 hr (Fig. 7). This result implies that constitutively expressed GBF can *trans*-activate *CP2*, although additional developmental signals are required for maximal expression. To address the nature of this requirement, we tested the cAMP-inducibility of *CP2* in A15-GBF cells in shaking culture. After 6 hr of starvation, wild-type cells respond to micromolar cAMP with a slow rise in *CP2* mRNA levels, from <2% after 1 hr to 100% after 6 hr. By contrast, treatment of 6 hr-starved A15-GBF cells with cAMP for 1 hr activates *CP2* to ~35% of the starved + 6 hr cAMP levels for either wild-type or A15-GBF cells. This rapid activation is consistent with the hypothesis that GBF is a limiting component of the signaling system that activates *CP2* in response to high, continuous levels of cAMP (see Discussion).

EcmA and *SP60* expression begins after that of primary late genes such as *CP2* and *DdrasD* (Fig. 7). Both of these transcripts are completely absent from *gbf*^{-/-} cells, indicating an absolute requirement for GBF and/or developmental signals dependent on GBF. cAMP added for 6 hr to cells under slow-shake conditions, which activates *ecmA* and *SP60* in wild-type cells, cannot bypass the requirement for GBF (Fig. 8). In A15-GBF cells, the developmental expression of *ecmA* and *SP60* is accelerated by ~2 hr (Fig. 7). The absence of these transcripts in

vegetative A15-GBF cells demonstrates that, in addition to high levels of GBF, other signals are required for their expression. Unlike *CP2*, however, this requirement cannot be met by cAMP alone, as the expression of these genes in A15-GBF cells still requires a 6-hr slow-shake incubation in the presence of cAMP (Fig. 8). Constitutive GBF expression may accelerate the fulfillment of this requirement however, as the slow + cAMP levels of *SP60* and *ecmA* transcripts are 2- and 20-fold higher, respectively, in A15-GBF than in wild-type cells.

The developmental kinetics of early-aggregation gene expression is largely unaffected in either *gbf*^{-/-} or A15-GBF cell lines (Fig. 7). However, in A15-GBF cells, levels of *D2* transcript are higher in late development and *csA* expression in early development is repressed. The abundant expression of *D2* and *csA*, as well as the weak expression of *CP2* transcripts, at 20 hr in *gbf*^{-/-} cells may be due to these cells re-expressing early gene markers after their initial failure to proceed through development. This effect is concurrent with the observed loosening of *gbf*^{-/-} aggregation mounds.

Discussion

The transition between early aggregation and later morphogenesis is one of the most important, yet poorly understood, developmental changes in *Dictyostelium*. It is at this stage that a loosely adherent mound of cells, having chemotaxed to a central position, differentiate to form a coherent organism of distinct cell types surrounded by a sheath. The product of the *GBF* gene, cloned here, is clearly a critical factor in this developmental switch. Disruption of the *GBF* gene arrests development at the loose aggregate stage, and although it has little effect on the expression of genes involved in

early aggregation, it virtually eliminates the activation of cell-type-specific late gene markers.

Because of the correlation between the affinity of a CA/GT-rich element for GBF in vitro and its ability to allow cAMP and developmental induction in vivo (see introductory section), we expect that GBF acts as a transcription factor that responds to an extracellular cAMP signal at these sites. As such, it would offer the first opportunity to study signal transduction in *Dictyostelium* by working backwards from the level of gene activation. That GBF function is cell autonomous is consistent with this hypothesis, as is the accelerated developmental activation of late gene markers in A15-GBF cells. In addition, the lack of induction of *DdrasD* and *CP2* (expressed before the high mound stage in wild-type cells) in *gbf*⁻ null cells cannot simply be the result of developmental arrest, indicating that GBF has a more direct effect on their transcription. GBF is also regulated in a manner that one might expect for a late gene activator. GBF transcript is present at low levels in starved cells and is activated as fast or faster than all tested late gene transcripts in response to cAMP. Allowing for a short delay for translation, the level of GBF activity in extracts mirrors GBF mRNA levels under all tested conditions. In accordance with these data, there is a correlation between *CP2* transcriptional activation (Hjorth et al. 1989) and GBF mRNA accumulation, which both reach near-maximal levels within an hour after stimulation by cAMP. Furthermore, overexpression of GBF results in the rapid activation of *CP2* in response to a 1-hr incubation with cAMP (~20-fold higher than wild type). Although it is possible that constitutive expression of GBF causes secondary effects that allow this enhanced response to cAMP, it seems most likely that GBF is the limiting factor in this activation. Combined with the correlation between in vitro GBF binding and in vivo activation by cAMP (see introductory section) these results strongly indicate that GBF is functioning as a transcriptional activator, at the end of a signaling pathway responsive to high concentrations of cAMP.

What might be the components of a signaling pathway that results in GBF *trans*-activation in response to cAMP? Pharmacological studies indicate that late genes are activated by a cell-surface cAMP receptor of ligand specificity similar to the receptor responsible for chemotaxis in aggregation and distinct from the specificity of cAMP-dependent protein kinase (Schaap and van Driel 1985; Gomer et al. 1986b; Oyama and Blumberg 1986; Haribabu and Dottin 1986). Of the cloned genes encoding cAMP receptors, only *cAR1* (required for chemotaxis) has a null phenotype consistent with a potential requirement for GBF activation by cAMP (Sun and Devreotes 1991), as *car3* nulls demonstrate wild-type development (Johnson et al. 1993) and *car2* nulls form tight aggregates and express late gene markers (Saxe et al. 1993). Of the G-proteins thought to be linked to the cARs, only $G\alpha 2$ has a null phenotype (similar to that of *cAR1* null cells) consistent with a potential requirement for GBF activation (for review, see Kimmel and Firtel 1991).

Cell-surface cAMP receptors are responsible for the

immediate, potential second-messenger responses to cAMP in aggregating cells, including adenylyl cyclase and guanylyl cyclase activation, and calcium influx. Of these, evidence suggests that the G-protein coupled activation of adenylyl and guanylyl cyclases are not required for late gene expression (Schaap et al. 1986; Kumagai et al. 1988; Haribabu et al. 1991). This makes sense, as these responses are adaptive and down-regulated by the continuous cAMP levels that activate late genes. The lack of a requirement for adenylyl cyclase activation renders the involvement of cAMP-dependent protein kinase PKA unlikely, especially because activation of *DdrasD* by cAMP is unaffected in cells lacking the PKA catalytic subunit (Mann and Firtel 1991). Calcium influx in response to cAMP is developmentally regulated (peaking at ~4 hr of starvation in shaking culture), and is a non-adaptive response, making it a possible candidate for GBF activation (Bumann et al. 1984). Experiments using calcium antagonists indicate that cAMP-induced Ca^{2+} influx is required, to differing extents, for late gene expression (Schaap et al. 1986; Blumberg et al. 1988, 1989; Kumagai et al. 1988). Finally, mutants with similar developmental phenotypes to *gbf*⁻ cells might be defective in a gene involved in GBF activation and/or nearby upstream or downstream events. One such mutant, *LagC*, also results in a loose aggregate phenotype and fails to activate many late genes (J. Dynes, A. Clark, G. Shaulsky, A. Kuspa, W. Loomis and R. Firtel, in prep.).

How might GBF function to activate promoters in response to cAMP? Because GBF-binding sites are required for the expression of both prestalk and prespore promoters, and GBF is present in both cell types, it is likely that GBF is not involved in cell-type choice. This implies that other promoter elements will be required for cell-type specification and that they work in concert with GBF-binding sites. Accordingly, two prespore promoters, *SP60* and *PspB*, require the combination of GBF-binding sites and an additional A/T element for their expression (Powell-Coffman and Firtel 1994; J. Powell-Coffman, G. Schnitzler, and R. Firtel, in prep.). Other studies also indicate that GBF cannot function alone as, unlike large promoter fragments, multimerized GBF binding sites are incapable of conferring late-developmental expression to disabled vegetative promoters (Pears and Williams 1988; J. Powell-Coffman et al., in prep.). Thus, GBF may only be able to act in synergy with other late-developmental transcription factors.

We propose a model for GBF function wherein GBF acts as a cell-type-nonspecific activator of late genes with GBF-binding sites in response to the continuous micromolar levels of extracellular cAMP that exist in aggregation mounds. The cAMP-activation of GBF probably works through the *cAR1* receptor and may involve increases in cytosolic calcium or second messengers downstream of $G\alpha 2$. We hypothesize that GBF can act immediately in the activation of the primary late genes that are induced early in the formation of the aggregate (such as *CP2*). The promoters of these genes may have been primed for activation by the binding of transcription factors responsive to early developmental signals

and are awaiting active GBF protein for maximal expression. Genes activated later in development may bind GBF as soon as it is produced but require additional (perhaps cell type specific) activators in conjunction with GBF. Many of these later transcription factors, and the signaling systems required to activate them, would be produced in the first wave of GBF-activated genes. Thus, GBF and the signaling pathways required to activate it are hypothesized to constitute a developmental switch between the formation of a crude multicellular aggregate and cellular morphogenesis in *Dictyostelium*.

Materials and methods

Extract preparation and GBF purification

Extracts of *Dictyostelium* cells were made as described in Hjorth et al. (1989) with the following modifications. Tris replaced HEPES (which interferes with GBF binding) in both lysis and extraction buffers. For nuclear extracts, nuclear pellets were treated as per Hjorth et al. (1990), extracts were brought to ~2.5 mg/ml protein and concentrated with 40% saturated ammonium sulfate, and the pellet was resuspended in and dialyzed against storage buffer [20 mM Tris (pH 8.4), 240 mM KCl, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, and 0.05% Triton X-100]. The cytosolic fraction was saved and centrifuged at 100,000g and dialyzed against storage buffer. Phenylmethyl sulfonyl fluoride (PMSF) (0.1 mg/ml), TPCK (0.033 mg/ml), TAME (0.1 mg/ml), pepstatin (1 µg/ml), aprotinin (2 µg/ml) and chymostatin (0.1 mg/ml) were used as protease inhibitors in the lysis buffer, and at 0.3× concentration in storage buffer. For extracts of GBF mutants and wild-type controls, PMSF, pepstatin, aprotinin, and chymostatin were added to the lysis buffer, but only the first three were included in the storage buffer. Extract was diluted fourfold with equalization buffer [6.7 mM Tris (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 0.05% Triton X-100 and 27 mM KCl] and passed over a sephadex G-25 column equilibrated in 80 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 DTT and 0.05% Triton X-100] to remove any material that might clog later columns. The flowthrough was applied to a DNA-cellulose column (Sigma) equilibrated in 80 mM KCl ACB, washed with 1 column volume each of 240 mM KCl ACB and 360 mM KCl ACB, and GBF was eluted with 2 column volumes of 1.1 M KCl ACB containing affinity column additives [ACAs; 10 mM MgCl₂, 1 mM spermine, 1 mM spermidine, 5 µg/ml of poly[d(I-C)], and 0.1% Tween-20]. Fractions with activity were pooled, dialyzed into ACB lacking KCl to bring the KCl to 134 mM and loaded on a CAE-1 affinity column (see below) equilibrated in 134 mM KCl ACB, and washed with 2 column volumes of 134 mM KCl ACB. A 4-column volume linear gradient from 134–600 mM KCl ACB was run and fractions containing GBF (peaking at ~290 mM KCl) were pooled and dialyzed into DEAE buffer [DEAE; 80 mM KCl, 5% glycerol, 20 mM Tris (pH 8.4), 0.05% Triton X-100, and 1 mM DTT]. This was loaded onto a DEAE-equilibrated DEAE-Sephrose CL-6B (Pharmacia) column with occasional stirring of the upper column bed, and the flowthrough (containing most of the GBF activity) and 135 mM KCl DEAE elution were collected.

Affinity column

The complementary oligonucleotides 5'-GATCTTTTTCACACACCCACACTAATTTACCCCATTTTG-3' and 5'-GATCCAAAAATGGGGTAAATTAGTGTGTGGGTGTGTGAA-

AAA-3', corresponding to the coding and transcribed strands (respectively) of CAE-1 from the *SP60* promoter (Haberstroh et al. 1991), were synthesized, annealed, ligated, and then coupled to Sepharose CL2B 300 (Sigma) as detailed previously (Kadonaga 1991). With a 61% coupling efficiency, the affinity resin was 43 µg/ml in CAE-1.

Mobility shift assays

Mobility shift assays were performed as described previously (Hjorth et al. 1989) with the following modifications. The sub-cloned *CP2* G-box 43-mer oligonucleotide (p0413) was isolated by digestion with *EcoRI* and *PstI* to produce an 84-bp fragment, and end-labeled with [α -³²P]dATP and Klenow. Typically 0.05 ng of labeled probe was incubated in a 20-µl reaction with 400 ng of poly[d(I-C)], 1 mg/ml of BSA, binding buffer, 3 ng of competitors, and extract (added last) for 15–30 min at room temperature (with modifications noted in the figure legends). In most cases, the final binding buffer was ¼× storage buffer, adjusted to account for storage buffer in extracts. At points in the purification, binding buffer was ACB 80–134 mM KCl, or DEAE or renaturation buffer (see below), and the differences in solute concentration and pH between these buffers do not greatly affect GBF binding (data not shown). The cloned *CP2* 43-mer and 43-mer mutant competitors (Hjorth et al. 1989) were isolated with *EcoRI* and *PstI*. The *SP60* CAE-1 oligonucleotide also binds GBF and functions as a strong competitor (J. Powell-Coffman, G. Schnitzler, and R. Firtel, in prep.), and was used as described in Haberstroh et al. (1991).

One unit of GBF activity is defined as the amount required to produce a 50% shift in the presence of 50 ng of poly[d(I-C)], as determined by laser densitometry. The number of apparent units in any mobility shift reaction is determined by comparison to a curve of titration with extract. Because the percent bound decreases roughly linearly with increasing log poly[d(I-C)], a correction can be made from apparent units at any given concentration of poly[d(I-C)] to units at 50 ng of poly[d(I-C)].

Renaturation of activity from SDS-PAGE

Elution and renaturation were performed as described previously (Hager and Burgess 1980) with modifications. Briefly, SDS-PAGE lanes containing GBF fractions were stained with Coomassie blue, size ranges or specific bands were excised, crushed with a glass Dounce homogenizer into elution buffer [EB; 0.1% SDS, 50 mM Tris (pH 7.9), 0.1 mM EDTA, 5 mM DTT, 0.1 mg/ml of BSA, and 150 mM KCl], and incubated overnight on a rolling platform. Gel fragments were spun down, and the supernatant was precipitated with 4 volumes of cold acetone, washed with 80% acetone/20% EB lacking SDS, resuspended in 6 M guanidinium hydrochloride renaturation buffer [RB; 10 mM Tris (pH 8.0), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, 0.05% Triton X-100, and 0.1 mg/ml of BSA], incubated at room temperature for 1 hr, diluted 50-fold with ice-cold RB and incubated on ice 2 hr before use in a mobility-shift assay. Recovery of binding activity was ~1%.

Isolation of peptides and microsequencing

DEAE flowthrough and 135 mM KCl fractions (containing 144,000 units of GBF activity, or ~36 µg protein) were precipitated with 13% TCA, washed with acetone, resuspended in 1× SDS-PAGE loading buffer, run on a 7.5% SDS-PAGE gel, and electroblotted to PVDF membranes (Immobilon-P, Millipore). The filter was stained with amido black, and excised bands were digested with trypsin. Released peptides were isolated by HPLC, and selected fractions were sequenced as described previously

(Fischer et al. 1991). To control for possible contamination of the abundant 37-kD protein (Fig. 1B), only those HPLC peaks present in the 92-kD band profile and not in the 37-kD band profile were selected for microsequencing.

PCR and cDNA cloning

Sense and antisense pairs of degenerate oligonucleotides were made to correspond to the regions of highest confidence for three of the peptide sequences. PCR reactions with paired sense and antisense oligonucleotides were performed as described previously (Mann and Firtel 1991) with CsCl-purified *Dictyostelium* genomic DNA. A combination of the 1037 sense oligonucleotide [5'-GTCTGGATCCGC(A/T/C)TT(T/C)AC(A/T/C)CC(A/T)GG(A/T/C)AT(A/T/C)ACNAA] and 1035 antisense oligonucleotide [5'-GTCTCTGCAGCNCNC(C/A)(A/C)NGGNGT(A/G)AANGG] resulted in an ~480 bp product at 48 and 53°C annealing temperatures. These reactions were extracted with phenol/CHCl₃, ethanol precipitated, and digested with *Bam*HI and *Pst*II, followed by phenol/CHCl₃ extraction, ethanol precipitation, and direct ligation into pSP72 (Promega). The specificity of the subcloned PCR product GBF-12 was confirmed by sequencing with Sequenase (U.S. Biochemical Corp.). A λZAP (Stratagene) cDNA library was prepared from RNA isolated from *Dictyostelium* cells developed for 12–16 hr as per the manufacturer's instructions and was screened using the PCR product and/or cDNA fragments from earlier screens as probes, to yield multiple overlapping cDNA clones.

General molecular biology and culturing of *Dictyostelium* strains

These experiments used the wild-type axenic strain KAX-3 and its thymidine auxotrophic derivative JH10 (Mann and Firtel 1991; Hadwiger and Firtel 1992). Procedures were performed as described in the following references, with any modifications noted: *Dictyostelium* culturing, development, and DNA and RNA isolation (Nellen et al. 1987), with 100 μM thymidine added to the HL5 medium of untransformed JH10 cells; fast- and slow-shake experiments (Mehdy et al. 1983; Mehdy and Firtel 1985); electroporation (Dynes and Firtel 1989), with neo^R transformants maintained in 15 μg/ml G418; β-galactosidase staining (Haberstroh and Firtel 1990) with modifications described in Powell-Coffman and Firtel (1994); Percoll gradients (Kubahara et al. 1993). Southern and Northern blots were probed with random-primed probes (Powell et al. 1992). The L9 gene-disruption construct was digested with *Eco*RV and *Apa*I before electroporation. A T3-TnT-coupled in vitro transcription/translation kit (Promega) was used as per the manufacturer's instructions, except that plasmid was linearized with *Apa*I before use.

Plasmid constructs

The gene-disruption construct (L9) was made by insertion of an ~3-kb fragment containing the *Thy1* gene (Dynes and Firtel 1989) into GBF cDNA sequences (encompassing the sequences between 24 and 2424 bp 3' of the initiation codon) between the *Hinc*II and *Eco*RI sites (678 and 1403 bp 3' of the ATG, respectively), in the pBluescript SK⁻ (pBSK⁻; Stratagene) vector (Fig. 2). The *Dictyostelium* A15-GBF expression construct (N27) was made by placement of cDNA sequences encompassing the complete GBF ORF (between 45 bp 5' and 2424 bp 3' of the initiation codon) behind the *Act15* promoter in the EXP4⁺ *Dictyostelium* expression vector (J. Dynes, A. Clark, G. Shaulsky,

A. Kuspa, W. Loomis, and R.A. Firtel, in prep.). To construct the transcription/translation plasmid (R20) the *Pst*II-*Kpn*I fragment from N27 (containing 5' GBF sequences) was subcloned into pBSK⁻. Approximately 15 ng of miniprep DNA from this subclone was amplified by PCR at an annealing temperature of 37°C, with the T7 promoter primer and an oligonucleotide [5'-GTTTGAGCTCTAGACACCATGCTGTCAACTCATCATCAAGG] designed to optimize the region surrounding the GBF ATG for translation in reticulocyte lysates. The ~750-bp product was digested with *Xba*I and *Kpn*I, purified (see above for details), and cloned, along with the 1.7-kb *Kpn*I-*Xho*I N27 fragment (containing 3' GBF sequences), into the *Xba*I-*Xho*I sites of pBSK⁻. The *Act15-lacZ* construct is described in Mann and Firtel (1993).

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G R Schnitzler, W H Fischer and R A Firtel

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