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Starvation promotes *Dictyostelium* development by relieving PufA inhibition of PKA translation through the YakA kinase pathway

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SUMMARY

When nutrients are depleted, *Dictyostelium* cells undergo cell cycle arrest and initiate a developmental program that ensures survival. The YakA protein kinase governs this transition by regulating the cell cycle, repressing growth-phase genes and inducing developmental genes. YakA mutants have a shortened cell cycle and do not initiate development. A suppressor of *yakA* that reverses most of the developmental defects of *yakA*$^-$ cells, but none of their growth defects was identified. The inactivated gene, *pufA*, encodes a member of the Puf protein family of translational regulators. Upon starvation, *pufA*$^-$ cells develop precociously and overexpress developmentally important proteins, including the catalytic subunit of cAMP-dependent protein kinase, PKA-C. Gel mobility-shift assays using a 200-base segment of PKA-C's mRNA as a probe reveals a complex with wild-type cell extracts, but not with *pufA*$^-$ cell extracts, suggesting the presence of a potential PufA recognition element in the PKA-C mRNA. PKA-C protein levels are low at the times of development when this complex is detectable, whereas when the complex is undetectable PKA-C levels are high. There is also an inverse relationship between PufA and PKA-C protein levels at all times of development in every mutant tested. Furthermore, expression of the putative PufA recognition elements in wild-type cells causes precocious aggregation and PKA-C overexpression, phenocopying a *pufA* mutation. Finally, YakA function is required for the decline of PufA protein and mRNA levels in the first 4 hours of development. We propose that PufA is a translational regulator that directly controls PKA-C synthesis and that YakA regulates the initiation of development by inhibiting the expression of PufA. Our work also suggests that Puf protein translational regulation evolved prior to the radiation of metazoan species.

Key words: Differentiation, Starvation, Cell cycle, *minibrain*, *pumilio*, *Dictyostelium*

INTRODUCTION

Nutrient depletion is probably the most common stress that cells encounter and must respond to in order to survive. This is true not only for heterotrophic microorganisms in their natural environment, but also for cells within multicellular animals (e.g., Jang and Hill, 1997). Starvation elicits numerous changes in physiology as cells attempt to re-establish homeostasis. If the starvation conditions persist, the cell may adapt by carrying out a differentiation program that generates new cellular functions appropriate to the new conditions. In many soil microorganisms, such as the yeasts and *Dictyostelium*, the developmental response to starvation is sporulation. These starvation responses share similar regulatory features such as cell cycle arrest and the expression of subsets of stress response proteins needed for survival or differentiation (Thevelein, 1994; Yamamoto, 1996; Maeda, 1997).

We have recently reported that YakA regulates the transition from growth to development in *Dictyostelium* (Souza et al., 1998). YakA is induced by starvation and is part of a pathway that links nutrient sensing to growth control and the initiation of development. YakA belongs to a subfamily of serine/threonine protein kinases including Yak1p from budding yeast and the Yak-related and minibrain kinases that have been characterized in a number of metazoan species, including humans (Garrett and Broach 1989; Tejedor 1995; Smith et al., 1997). Members of this conserved protein family may be components of a conserved regulatory system governing growth/differentiation transitions in eukaryotes. Relative to wild-type cells, *yakA*$^-$ mutants have a faster cell cycle and smaller cell size and are unable to initiate development. The overexpression of *yakA* results in cell cycle arrest and rapid early development. A function in cell cycle arrest and cell survival has also been described for Yak1p in yeast where it is part of the response to conditions that activate the Msn stress-response transcription factors (Smith et al., 1998). Yak1p is induced by cell cycle arrest and induces thermotolerance (Garrett et al., 1991; Hartley et al., 1994). Thus, in both *S. cerevisiae* and *Dictyostelium*, Yak kinases allow cells to respond to environmental stress by initiating a differentiation program.

In *Dictyostelium*, nutrient sensing is mediated by prestarvation factor (PSF), a secreted protein that allows cells to monitor their cell density in proportion to their bacterial food source (Rathi et al., 1991; Clarke and Gomer, 1995). Prior to overt starvation, PSF signaling results in the limited induction of some genes required for early development. There is some evidence that YakA is an effector of the PSF starvation response pathway (Souza et al., 1998). Under conditions of gradual food depletion, YakA expression parallels the activity of PSF, increasing from a low basal level to a maximum level when the food is exhausted. YakA expression is also induced by a factor released by starving cells that is likely to be PSF. In *yakA*$^-$ cells, PSF production is normal and the induction of one PSF-responsive gene, *dscA*, is normal. Since YakA appears to control cell division during growth by ensuring that cells are the proper size before they divide, the regulation of YakA by PSF might provide a way for cells to coordinate nutrient availability with cell division.

After *Dictyostelium* cells sense starvation, YakA appears to operate in part by inducing a 5-fold increase in the catalytic subunit of cAMP-dependent protein kinase (PKA-C), which normally occurs at the onset of development (Souza et al., 1998). PKA-C appears to be a key control point for YakA-mediated regulation since the constitutive expression of PKA-C restores development to *yakA*$^-$ cells but YakA expression does not restore development to *pkaC*$^-$ cells. PKA has been extensively characterized in *Dictyostelium* and has been implicated in the regulation of both early and late gene expression, the timing of cAMP production and cell differentiation and the coordination of fruiting body morphogenesis with the terminal differentiation of spores and stalk cells (reviewed by Firtel, 1996; Loomis, 1998). At the start of development, PKA-C is required for the expression of key cAMP signaling proteins such as the aggregation-stage adenylyl cyclase, ACA, and the major cAMP receptor, cAR1 (Schulkes and Schaap, 1995; Mann et al., 1997). After 4-6 hours of development, robust cAMP signaling begins and cAMP serves as a chemotactic signal that controls aggregation of the cells into a multicellular organism. The requirement for PKA-C in initiating development cannot be solely ascribed to its role in mediating ACA expression, however, since the constitutive expression of ACA does not restore the development of *pkaC*$^-$ cells (Mann et al., 1997). Thus, there are likely to be other functions activated by PKA-C that are required for aggregation. After starvation, the amount of PKA-C appears to determine the timing of later events; higher than normal levels of PKA-C speed development, whereas inhibition of PKA-C slow or halt development (Firtel and Chapman, 1990; Simon et al., 1992; Mann et al., 1992; Anjard et al., 1992; our own unpublished observations). One plausible model for the growth to development transition is that YakA mediates the starvation response by inducing the production of sufficient PKA-C to initiate development.

The regulatory pathway that leads from the activation of YakA to the acquisition of developmental competence remains obscure. In view of the importance of the Yak/minibrain kinases in cellular differentiation, including within the developing mammalian brain, we have begun to dissect YakA-mediated regulation. We have identified additional components of YakA signaling by isolating genetic suppressors of the developmental deficiency of *yakA*$^-$ cells. We have characterized one such component, PufA, which mediates part of the YakA response. The Puf proteins studied in *Drosophila* and *C. elegans* are regulators of translation and are characterized by the presence of a sequence-specific RNA-binding domain at the C terminus, the pumilio homology domain or PUM-HD (Murata and Wharton, 1995; Zhang et al., 1997; Wharton et al., 1998; Zamore et al., 1997). The *Drosophila* Pumilio protein inhibits the translation of hunchback mRNA in a way that spatially limits the production of this transcriptional repressor of abdomen-specific genes (Murata and Wharton, 1995). FBF-1 and FBF-2 in *C. elegans* control the translation of FEM-3, a protein that regulates the switch from sperm to oocyte production within the hermaphrodite gonad (Zhang et al., 1997). Both the hunchback and *fem-3* mRNA contain 3′-UTR elements that are recognized by Pumilio and the FBFs, respectively, which are required for the translational inhibition mediated by these proteins (Murata and Wharton, 1995; Ahringer and Kimble, 1991).

Here, we report that one of the targets of PufA regulation is PKA-C. We also provide evidence that PufA binds to a region in the 3′ end of the *pkaC* mRNA, which is consistent with a role for PufA in controlling PKA-C translation. In addition, we show that YakA is required to shut off PufA expression at the onset of development. This supports our earlier findings that YakA is a regulator of the switch between vegetative and developmental gene expression and provides a plausible explanation for how mutations in *pufA* suppress the developmental phenotype of *yakA*$^-$ cells. Our results suggest that YakA mediates the starvation response in *Dictyostelium*, in part, by repressing PufA expression, which relieves the negative control on PKA-C expression and leads to the onset of development.

MATERIALS AND METHODS

Growth, development and transformation of *Dictyostelium*

All strains were derived from the axenic *Dictyostelium discoideum* strain AX4 (Knecht et al., 1986) and were grown in axenic media (HL-5) or on SM agar plates in the presence of *Klebsiella aerogenes* (Sussman, 1987). Growth curves were determined in HL-5. Five clones of each strain were grown to 1×10^6 cells/ml, diluted to 5×10^5 cells/ml and counted using a hemocytometer. Growth curves for mutants were determined in side-by-side tests with non-mutant sibling transformants. Cells were developed on Millipore filters as described (Sussman, 1987) and photographed with an Optronics CCD camera. Cell size measurements were obtained as previously described (Souza et al., 1998). Spore tests were performed as described (Wang et al., 1999).

REMI mutagenesis (Kuspa and Loomis, 1992) was carried out with the *Bam*HI-linearized plasmid pBsr1, and the restriction enzyme *Dpn*II, according to Adachi et al. (1994). Transformants were selected in HL-5 supplemented with 4 μg/ml Blasticidin. Strains that expressed the putative *pkaC* PRE elements were obtained by calcium-phosphate-mediated transformation and selection with Geneticin (Nellen et al., 1987).

The screen for mutations that suppress the development phenotype of *yakA*$^-$ cells was carried out as follows. The *yakA*$^-$ mutant AK800, which harbors a plasmid insertion (IS800) in the sequence that encodes the protein kinase core (Souza et al., 1998), was used as the parental strain for insertional mutagenesis. A REMI-mutagenized population of 4,000 clones was plated in the presence of *Klebsiella*

aerogenes and approximately 10,000 colonies were visually inspected for the formation of multicellular structures. One mutant (AK804) was observed to undergo development and isolated for further study.

DNA and RNA manipulations

Standard DNA and RNA manipulations were carried out as described (Sambrook et al., 1989). Flanking genomic DNA was recovered from the AK804 genome by plasmid rescue using *Eco*RI to liberate a 9 kb fragment, which was cloned as described (Kuspa and Loomis, 1994), to generate the plasmid p804Eco. Using the genomic insert from p804Eco as a hybridization probe on Southern blots, a 5 kb *Eco*RI fragment in wild-type DNA, and a 9 kb *Eco*RI fragment in AK804 DNA, was observed. Disruption of the *pufA* gene by homologous recombination using *Eco*RI-digested p804Eco and selection for blasticidin S-resistance was carried as described (Kuspa and Loomis, 1994; Adachi et al., 1994). Southern analysis confirmed that transformants that integrated the fragment into the original insertion site (IS804) had the same developmental phenotype as AK804, whereas transformants that integrated the plasmid elsewhere were wild type. The plasmid rescued from AK804 contained 2 kb of genomic DNA upstream of the insertion site with an opening reading frame that ended at the *Eco*RI site with no apparent start codon. The remaining 5′ of the gene was cloned as a 4 kb *Bam*HI fragment isolated from a partial genomic library generated in the plasmid pGEM3 (Promega, Wisconsin).

RNA samples were prepared from cells developing on Millipore filters. RNA was extracted using the Trizol reagent as described by the manufacturer (Life Technologies), subjected to electrophoresis in 1.2% agarose/formaldehyde gels and transferred to nitrocellulose filters as described (Sambrook et al., 1989). The DNA fragments used as probes were as follows: a *Bam*HI-*Xho*I fragment containing the full-length cDNA of *cprD*, an *Eco*RI fragment containing the full-length *acaA* cDNA and a *Bam*HI-*Hind*III fragment of *pkaC* that excludes the non-conserved repeats at the N terminus of the protein. Equal loading of RNA in the different gel lanes was confirmed by ethidium bromide staining and by hybridization of the filters with the constitutively expressed gene IG7 as a probe (Early and Williams, 1988).

The RNAse protection assay was performed using the RPA II Ribonuclease Protection Assay Kit (Ambion, Inc.) and analyzed using denaturing conditions according to the manufacturers instruction. Control experiments confirmed that all reactions were performed with a 10-fold excess of probe RNA. The antisense riboprobe for *pufA* RNAse protection assays was obtained using the Riboprobe Combination SP6/T7 kit (Promega) according to the manufacturers instruction. The template in these reactions was the 139 bp *Hinc*II-*Bam*HI fragment (bases 2080 to 2219 of the open reading frame) cloned into pGEM3. The sense and antisense probes for the $pkaC_{PRE}$ element was obtained by in vitro transcription using the Riboprobe kit (Promega). The template for both sense and antisense riboprobes was a 202 bp fragment corresponding to *pkaC* PREs. The fragment corresponds to bases 1838 to 2040 of the *pkaC* open reading frame. The same fragment was cloned into pDNeo67 (da Silva and Klein, 1990) for expression experiments in *Dictyostelium* cells.

RNA-binding assays

RNA-binding assays were carried out as described (Murata and Wharton, 1995). Briefly, 2 μg of nickel-agarose purified protein (see below) were incubated with 5×10^5 counts/minute of *pkaC* PRE sense or antisense riboprobes in 10 mM Hepes pH 7.5, 5 mg/ml heparin, 1 mM DTT, 10 μg/ml yeast tRNA, 0.1 mg/ml poly(rU), 10 U RNAsin in a final volume of 10 μl. Following incubation at room temperature for 10 minutes, 2 μl of 50% glycerol was added and the reactions were electrophoresed through a 5% non-denaturing polyacrylamide gel containing 5% glycerol and 0.5× TBE. Electrophoresis was performed for 4 hours at 4°C, and the gel was dried and exposed to X-ray films.

Protein manipulations

Protein extracts were prepared by freezing and thawing frozen cell pellets in 10 mM Tris (pH 7.8) containing 4 μg/ml pepstatin, 4 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. The extracts were clarified by centrifugation at 12,000 ***g*** for 10 minutes and blotted to nitrocellulose filters using a slot-blot apparatus. Alternatively, the samples were submitted to SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose filters as described (Laemmli, 1970; Harlow and Lane, 1988). Immunological detection of PKA-C was accomplished by incubation of the blots with rabbit anti-PKA-C antibodies (generously provided by M. Veron and F. Traincard). The crude antiserum was diluted 1:1000 in 10 mM Tris, 150 mM NaCl, 0.1% Tween-20 containing 1 % bovine serum albumin and incubated with the blots overnight at 4ºC. Immunodetection was performed with horseradish peroxidase-conjugated goat anti-rabbit antibodies using the ECL Western Blotting analysis system (Amersham).

PufA was partially purified from *Dictyostelium* cell extracts by absorption to nickel-agarose columns. Briefly, cells were harvested at different times of development, or during exponential growth, and lysed in 20 mM sodium phosphate, 500 mM sodium chloride pH 7.8 containing 4 μg/ml pepstatin, 4 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Clarified extracts were bound to nickel-agarose columns. The columns were washed in 20 mM sodium phosphate, 500 mM sodium chloride pH 6.0 and bound protein was sequentially eluted with wash buffer containing 200 and 500 mM imidazole. Eluate fractions were analyzed by polyacrylamide gel electrophoresis and western blotting, with the Penta-His antibody (Qiagen), to identify the eluted proteins, and were used in RNA-binding experiments. Protein was estimated using the Bio-Rad Protein Assay (Bio-Rad Laboratories).

Biochemical analyses

PKA activity measurements were carried out using the SignaTECT PKA assay system (Promega). Samples were prepared from cells that were growing in HL-5 liquid media or cells that were developing on Millipore filters. Cell extracts containing 10 μg of protein were prepared according to the manufacturers instructions and were used in reactions in the presence of 10 μM cAMP and in the presence or absence of 10 mM of the PKA-specific inhibitor PKI, which inhibits the *Dictyostelium* enzyme (Mann et al., 1992). PKA activity was defined as the amount of Kemptide substrate phosphorylated (nmol/min/mg protein) in the absence of PKI, minus the amount phosphorylated in the presence of PKI.

DNA and protein sequence analyses

The *pufA* sequence was compared to the sequences present in the databanks using the BLAST search program from the National Center of Biotechnology Information. An alignment of the protein sequences was obtained using the multiple alignment search tools at the Human Genome Center, Baylor College of Medicine, Houston, TX. The PufA amino acid and nucleotide sequences have been deposited in GenBank under the accession number AF128626.

RESULTS

Mutations in *pufA* suppress the developmental deficiency of *yakA*⁻ cells

YakA is a protein kinase that is essential for proper cell cycle control and for the initiation of development (Souza et al., 1998). *yakA*⁻ cells are smaller than wild-type cells, divide more rapidly and, upon starvation, do not aggregate. The expression of genes that are normally induced by starvation, like those encoding the cAMP receptor cAR1 or the adenylyl cyclase ACA, is absent in this mutant. To identify potential effectors

of YakA, we used insertional mutagenesis to isolate suppressors of the *yakA* mutant phenotype. We subjected *yakA*$^-$ cells to restriction enzyme-mediated integration (REMI) with linearized plasmid DNA and screened the resulting mutagenized clones for their capacity to undergo development (see Materials and Methods). One mutant was able to produce multicellular structures and was found to have an insertion in a gene that we named *pufA*. The *yakA*$^-$ *pufA*$^-$ double mutants displayed accelerated development during the first hours after starvation compared to wild-type cells (Fig. 1). Wild-type cells formed tight aggregates after 12 hours of starvation, while *yakA*$^-$ *pufA*$^-$ cells formed aggregates by 10 hours. After *yakA*$^-$ *pufA*$^-$ cells formed standing fingers (12-16 hours), they became asynchronous in that most of them did not progress through the series of morphogenetic movements that normally produce a fruiting body by 24 hours. The fingers that were formed fell back to the substratum and some eventually formed normally proportioned fruiting bodies over the next 20 hours (not shown). Many of the aggregates, however, did not form fruiting bodies and remained as fingers and aberrant masses of cells.

The mutated gene, *pufA*, was isolated by plasmid rescue into *E. coli* and used to recreate the insertion mutation in other host strains by homologous recombination. Introduction of the *pufA* mutation into *yakA*$^-$ cells, or into wild-type cells, resulted strains with developmental phenotypes that were very similar to the original *yakA*$^-$ *pufA*$^-$ mutant isolate (Fig. 1). This demonstrates that the insertion event that inactivated the *pufA* gene caused the observed suppression of the *yakA*$^-$ phenotype in our screen. In addition, we have extended this screen to ~70,000 insertion mutations in *yakA*$^-$ cells and have isolated two additional suppressors within the *pufA* gene (G. Chen and A. K., unpublished data).

The fact that *pufA*$^-$ cells displayed precocious aggregation and aberrant finger structures similar to the double mutants indicates that *pufA* normally regulates the timing of development and terminal morphogenesis. However, it should be kept in mind that the late phenotypes may be an indirect result of the early timing defects. This collection of phenotypes is strikingly similar to that found in wild-type cells that overexpress *yakA* (Souza et al., 1998). This similarity, together with the suppression data, lead to the genetic inference that YakA normally initiates development by counteracting an inhibitory effect of PufA.

Inactivation of *pufA* does not rescue the growth defects of *yakA*$^-$ cells

Since YakA has been implicated in cell cycle control, we looked for possible effects of the *pufA* mutation on the growth phenotypes of wild-type and *yakA*$^-$ cells. To do this, we determined the doubling times and the sizes of wild-type, *yakA*$^-$, *yakA*$^-$ *pufA*$^-$ and *pufA*$^-$ mutant cells. Wild-type cells and *pufA*$^-$ cells doubled about every 8-10 hours during exponential growth, while *yakA*$^-$ and *yakA*$^-$ *pufA*$^-$ cells doubled every 6-8 hours (Fig. 2). Thus, mutating *pufA* does not alter the fast growth phenotype of *yakA* mutants and does not change the growth rate of wild-type cells. In addition, the *pufA* mutation has no effect on the size of the cells. The cross-sectional area of *yakA*$^-$ and *yakA*$^-$ *pufA*$^-$ cells was measured to be 74±3% and 77±3% of wild-type cells, respectively, while *pufA*$^-$ cells were 104±2% the size of wild-type cells. These results imply that the inactivation of *pufA* restores the ability of *yakA*$^-$ cells to initiate development, but does not alter the cell cycle defects associated with the absence of this kinase.

Inactivation of *pufA* restores developmental gene induction, but not growth-stage gene repression, in *yakA*$^-$ cells

The mRNA expression of the cysteine proteinase gene *cprD* rapidly declines at the onset of development (Souza et al.,

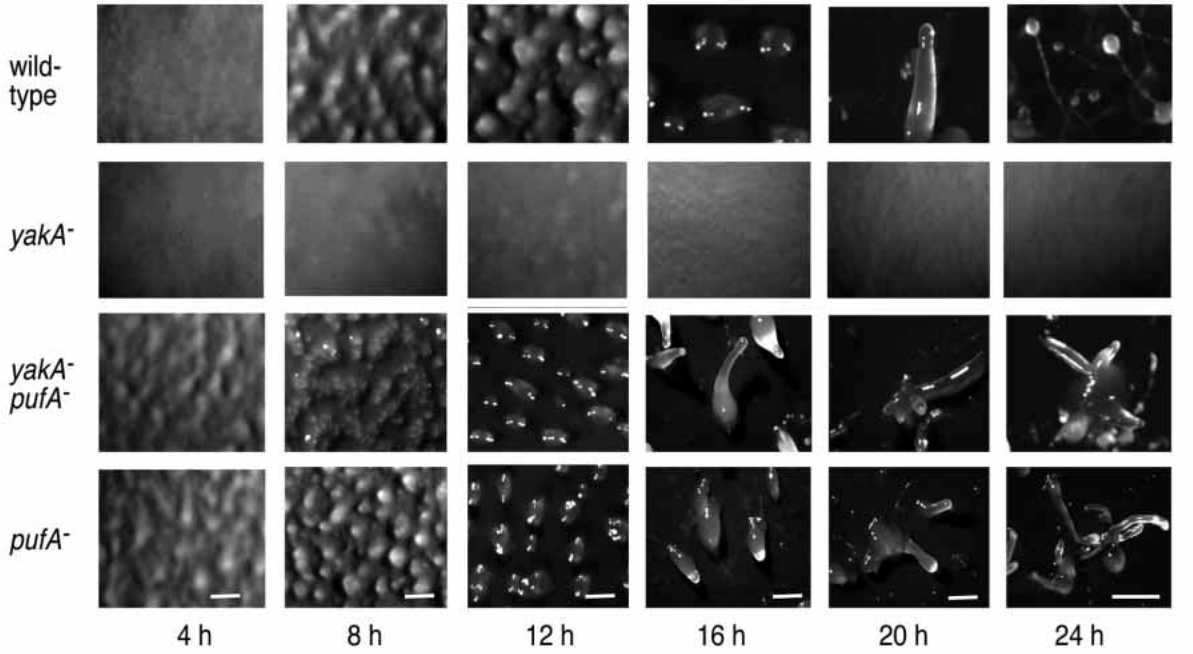

Fig. 1. Developmental phenotypes. Wild-type cells, *yakA*$^-$ mutants, *yakA*$^-$ *pufA*$^-$ double mutants and *pufA*$^-$ mutants were plated on Millipore filters and photographed after various times of development. Bars, 0.5 mm.

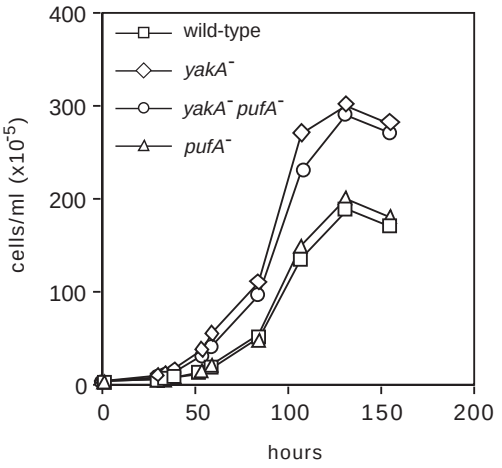

Fig. 2. YakA-null cells divide faster than wild-type cells. Growth curves of *yakA*⁻ *pufA*⁻ double mutant cells, sibling *yakA*⁻ cells, *pufA*⁻ cells and sibling wild-type control cells. Cells were grown in HL-5 liquid media and cell densities were determined by direct counting. Representative curves from greater than 5 comparisons of different isolates of each strain, carried out in parallel, are shown.

1995). We had previously observed that this decrease does not occur in *yakA*⁻ cells, indicating that YakA function is required to shut down the expression of some vegetative genes (Souza et al., 1995, 1998). The mRNA pattern for *cprD* in *yakA*⁻ *pufA*⁻ cells showed that *cprD* expression continues for up to 16 hours of starvation (Fig. 3). In wild-type and *pufA*⁻ cells, *cprD* mRNA decreased within 2 hours of starvation. This implies that *yakA*, but not *pufA*, is required for the decrease in vegetative gene expression observed in wild-type cells.

The inability of *yakA*⁻ cells to develop is reflected in the absence of developmental gene expression. *yakA*⁻ cells do not increase the steady-state mRNA level for the adenylyl cyclase gene, *acaA*, or for the PKA-C gene, *pkaC* (Fig. 3; Souza et al., 1998). In *yakA*⁻ *pufA*⁻ cells, the expression of *acaA* was restored, but it was induced earlier in development compared to wild-type cells (Fig. 3). The *pufA*⁻ cells also displayed precocious expression of *acaA* mRNA. The expression of *acaA* is normally restricted to the aggregation phase in wild-type cells (Pitt et al., 1992), but was expressed throughout development in *pufA*⁻ cells, as well as during growth (Fig. 3). The *pkaC* mRNA pattern is also altered in *yakA*⁻ *pufA*⁻ cells. Some restoration of *pkaC* expression occurred in *yakA*⁻ *pufA*⁻ cells relative to *yakA*⁻ cells, but not to the levels observed in wild-type cells. The *pkaC* expression in *pufA*⁻ cells was also reduced relative to wild-type cells although the pattern of expression was similar. At around 16 hours, there was a sharp rise in *pkaC* mRNA followed by a sharp decrease. This was most obvious in the *yakA*⁻ *pufA*⁻ cells, but also occurred in the *pufA*⁻ cells. Interestingly, this is the time in development when the *pufA* mutants became asynchronous and delayed in development.

YakA functions at the transition between growth and development and the results that we have presented so far indicate that *pufA* inactivation renders the initiation of development independent of YakA. The absence of observable growth phenotypes in *pufA*⁻ cells, and the inability of a *pufA* mutation to rescue the growth phenotype of *yakA*⁻ cells, suggests that PufA mediates at least one key development-specific function of YakA.

pufA encodes a putative regulator of translation

The identification and sequence analysis of the *pufA* gene revealed that it encodes a protein with a high degree of similarity to *Drosophila* Pumilio and Pumilio-related proteins from humans and *C. elegans*. These proteins have been named Puf proteins after the two family members for which there is substantial functional data, Pumilio and the FBFs from *C. elegans* (Zhang et al., 1997). The similarity between Puf proteins is restricted to the Pumilio homology domain (PUM-HD), usually found at the C terminus, which consists of 8 repeats of 32 amino acids each containing the consensus sequence D(Q/K)(F/Y)(A/G)NYV(V/I)QK near the center of each repeat, flanked by additional amino acids that are critical for function (Macdonald, 1992; Zhang et al., 1997; Fig. 4). Pumilio and the FBF proteins each regulate the translation of key developmental regulators by binding to the 3′ end of those regulators' mRNA. It has been demonstrated that the essential function of Pumilio and FBF can be carried out in vivo by the PUM-HD alone (Wharton et al., 1998; Zhang et al., 1997). The PUM-HDs of Pumilio and FBF have also been shown to be necessary and sufficient for binding to the 3′-UTR of the mRNAs that they regulate (Zhang et al. 1997; Wharton et al., 1998; Zamore et al., 1999). Thus, the presence of this domain suggests that PufA functions by binding to an mRNA and regulating its translation. An amino acid alignment of the PUM-HDs of

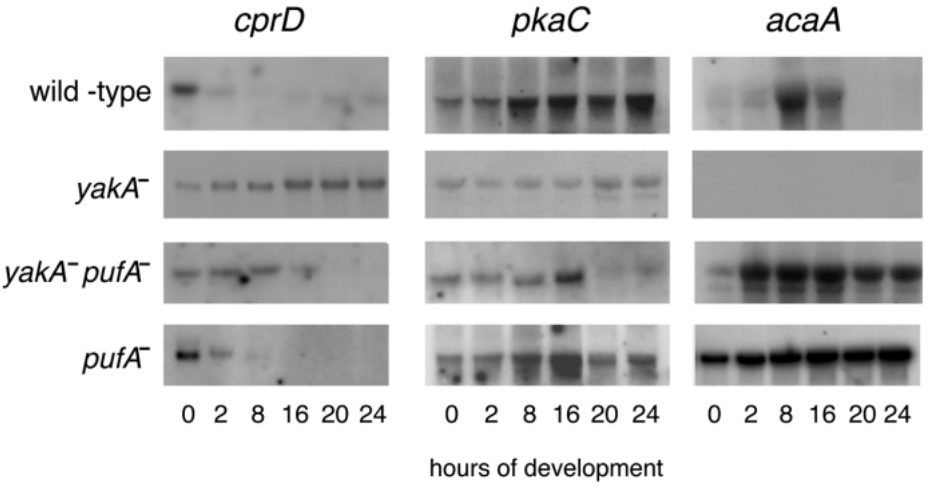

Fig. 3. Gene expression in *pufA*⁻ mutants. Wild type, *yakA*⁻ mutants, *yakA*⁻ *pufA*⁻ double mutants and *pufA*⁻ mutants were developed on nitrocellulose filters for the times indicated. Samples of total RNA (20 μg) were analyzed on northern blots using fragments of the indicated genes as hybridization probes.

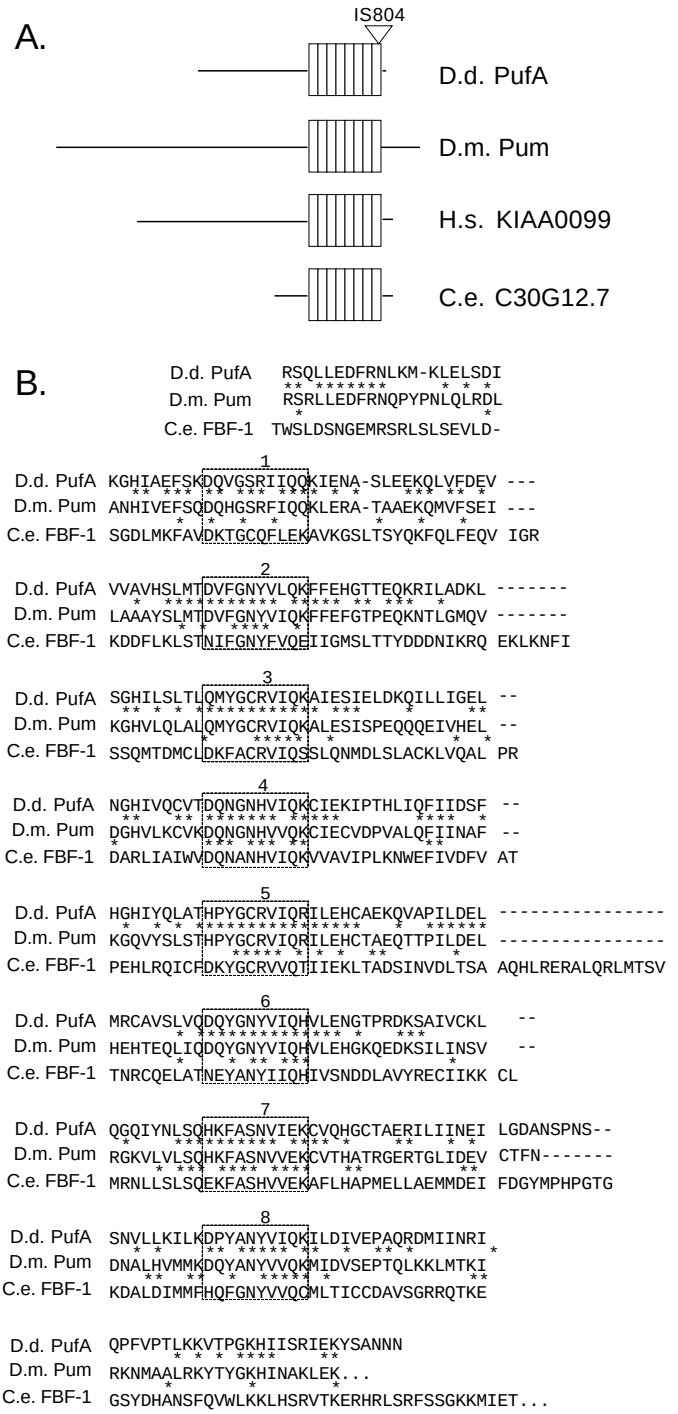

Fig. 4. Alignment of the predicted PufA protein with other Puf proteins. (A) A schematic depiction of PufA is compared to other Puf proteins. The boxes with vertical bars indicate the PUM-HD repeats and the triangle indicates the location of the insertion mutation found in AK804. (B) An alignment of the predicted amino acid sequences corresponding to the PUM-HD region of PufA and other Puf proteins. The numbered lines show the eight 36-amino acid repeats of the PUM-HDs and the dotted lines denote the highly conserved 10-amino acid core sequence within each repeat. D.m. Pum is the *Drosophila* pumilio protein (Macdonald, 1992) and C.e. FBF-1 is from *C. elegans* (Zhang et al., 1998). The predicted full-length PufA protein is in GenBank (accession number AF128626).

Pumilio, FBF-1 and PufA showed that PufA is more similar to Pumilio than it is to FBF-1 (Fig. 4B). PufA is 56% identical to Pumilio within the PUM-HD region (including 73/80 of the consensus amino acids), but is only 24% identical to FBF-1, while FBF-1 and Pumilio are 27% identical in their PUM-HD domains.

PufA controls PKA-C protein expression

The rapid aggregation and delayed fruiting body formation observed in developing *pufA*$^-$ cells are consistent with elevated PKA activity, as others have described for mutants in the regulatory subunit of PKA (PKA-R) or PKA-C overexpression, and as we have observed for YakA overexpression (Simon et al., 1992; Anjard et al., 1992; Mann et al. 1992; Souza et al., 1998). We have previously shown that the increase in PKA activity triggered by nutrient starvation is absent in *yakA*$^-$ cells and that overexpression of either YakA or PKA-C in these cells results in high constitutive PKA activity and restores aggregation (Souza et al., 1998). To test if the suppression of the *yakA* phenotype by the inactivation of *pufA* is due to the induction of PKA-C, we measured the PKA activity and PKA-C protein content in *yakA*$^-$ *pufA*$^-$ cells and *pufA*$^-$ cells. PKA activity is 2-fold higher during growth in *yakA*$^-$ *pufA*$^-$ cells, and in *pufA*$^-$ cells, relative to wild-type cells (Table 1).

Immunoblots of cell extracts using an antibody raised against PKA-C confirmed that the elevated activity was due to an increase in PKA-C protein (Fig. 5). Western blot analysis of cells harvested at 0, 8, and 16 hours of development show that *pufA*$^-$ cells and *yakA*$^-$ *pufA*$^-$ cells produced significantly more PKA-C than wild-type cells throughout development (Fig. 5A). *yakA*$^-$ cells were found to have much less PKA-C than wild-type cells, as predicted from the reduced *pkaC* mRNA in these cells. The low molecular weight polypeptides are probably breakdown products of PKA-C, since they were not detectable in *pkaC*$^-$ cells (not shown), and this suggests that degradation of this protein is proportional to the expression level. To quantitate PKA-C levels, we analyzed serial dilutions of cell extracts by immunological detection and densitometry. This revealed that *yakA*$^-$ *pufA*$^-$ cells and *pufA*$^-$ cells have 5-fold more PKA-C during vegetative growth than their parental counterparts (Fig. 5B). At 16 hours of development, both mutants have about 10-fold more PKA-C compared with wild-type cells. In addition, quantitation of northern blot data demonstrated that *pufA*$^-$ cells have about 2-fold less *pkaC* mRNA during growth and early development compared with wild-type cells, and about the same amount at 16 hours of development (data not

Table 1. Dependence of PKA activity on YakA and PufA

Strain	PKA activity* (pmoles ATP/min/mg protein)
Wild type	54±5.5
YakA$^-$	29±8.0
YakA$^-$*pufA*$^-$	90±8.5
PufA$^-$	130±11
Wild type[*act6:: pkaC*$_{PRE}$]	250±15

*The PKI-inhibited phosphorylation of Kemptide in the presence of 10 mM cAMP is shown. The values represent the mean and s.e.m. for three independent experiments with cells growing in HL-5.

shown). These results imply that *pkaC* mRNA is translated >10 times more efficiently in *pufA*⁻ cells compared to wild-type cells.

Mutants with increased PKA activity have been shown to form spores precociously during development (reviewed by Loomis, 1998). Consistent with this, *pufA*⁻ cells produce 150 times more spores during vegetative growth than wild-type cells. Only 0.001% of wild-type cells growing in HL-5 liquid media are spores, whereas 0.15% of *pufA*⁻ cells are spores under the same conditions. *pufA*⁻ cells produce about the same number of spores as wild-type cells after 36 hours of development (data not shown).

The *pkaC* mRNA contains putative Puf protein recognition elements

The results described above suggest that the inactivation of *pufA* rescues the development of *yakA*⁻ cells by causing increased synthesis of PKA-C. This genetic inference, together with PufA's sequence similarity to Puf proteins, predicts that PufA acts as a negative regulator of PKA-C mRNA translation. Since PufA is much more similar to Pumilio than it is to FBF-1 (Fig. 4B), we reasoned that potential Puf protein-binding sites present on the *pkaC* message would be more similar to the Pumilio recognition elements (NREs) than they are to the FBF recognition element (PME). The NREs found in the hunchback mRNA that are recognized by Pumilio have been defined by extensive in vitro binding studies and functional studies in vivo (Murata and Wharton, 1995; Wharton et al., 1998; Zamore et al., 1999). Although two similar NREs are found next to each other in the hunchback mRNA, only one of these is required for high affinity binding in vitro (Wharton et al., 1998; Zamore et al., 1999). Within each NRE two ribonucleotide triplets 'UGU', separated by 7 nucleotides, appear to be critical for Pumilio binding and function. One or two residues upstream and downstream of these triplets are also important for NRE function to varying degrees, depending on the assay used to assess function, and a minimum length of 26 nucleotides surrounding the UGU triplets is required for high affinity binding in vitro (Wharton et al., 1998; Zamore et al., 1999). Inspection of the *pkaC* mRNA revealed a region near the 3′ end of this message that contained several sequence elements that are similar to the NREs found in the hunchback mRNA. We will refer to these elements as putative Puf response elements (PREs). The alignment of the putative PREs in *pkaC* with the hunchback NREs show that many of the key residues required for the function of an NRE are present in the putative PREs (boxed residues in Fig. 6A).

To test if the putative PRE elements are likely to be recognized by a Puf protein, we performed gel mobility-shift assays using sense and antisense RNA probes representing the cluster of PREs found in 3′ terminus of *pkaC*. In initial experiments, we found that a complex was formed on the sense probe, but not on the antisense probe, when we used extracts made from wild-type cells, but not when the extracts were made from *pufA*⁻ cells. Although these results were consistent with a protein binding to the end of the *pkaC* mRNA, the complex was barely detectable in standard cell-free extracts (data not shown). Since the predicted PufA protein contains two stretches of histidines (7 and 5 residues long) that could be used for the purification by nickel absorption, we partially purified proteins from crude cell extracts by absorption and elution from nickel-agarose columns and tested those in the mobility-shift assay (see Materials and Methods). These eluates appear to contain PufA protein (see below). After this purification step, there was a dramatic improvement in our ability to detect the complex, indicating that the protein(s) involved in formation of the complex bind to nickel-agarose.

We explored the properties of the PRE-binding complex further by using protein eluates from nickel-agarose columns and the mobility-shift assay. Complex formation was observed with eluates derived from growing wild-type cells, while less complex was formed with eluates from developing cells (Fig. 6B). In addition, this complex was not detected in eluates derived from *pufA*⁻ cells. Interestingly, the highest amount of

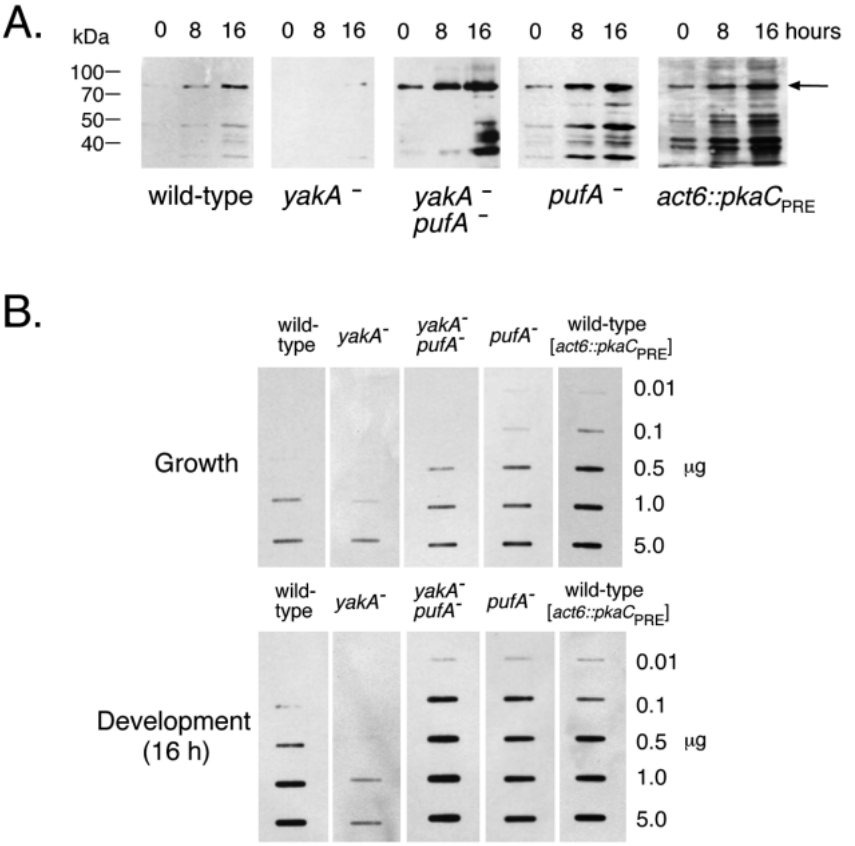

Fig. 5. Elevated expression of PKA-C protein in PufA mutants. (A) Western blot analysis using anti-PKA-C antibodies was carried out to detect PKA-C during growth and development. The arrow indicates the position of PKA-C. (B) Dilutions of cell extracts derived from the indicated strains, during growth or after 16 hours of development on filters, were applied to nitrocellulose filters and PKA-C was detected as in (A).

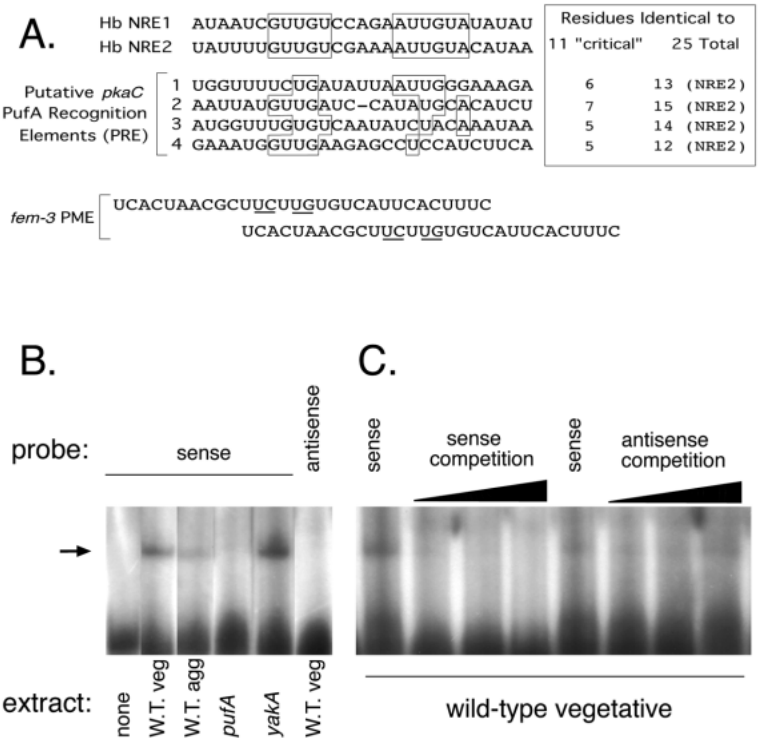

Fig. 6. Gel mobility shift of putative *pkaC* PRE depends on *pufA*. (A) Alignments of the putative PufA response elements (PREs) found in *pkaC* with the hunchback NREs are shown. The hunchback NREs shown are as defined by the functional studies of Wharton et al. (1998) and Zamore et al. (1999), with the critical residues boxed. All PREs fall between nucleotides 1,838 and 2,040 of the *pkaC* open reading frame (Burki et al., 1991; Mann and Firtel 1991). The critical residues for the PME are underlined (Ahringer and Kimble, 1991). (B) Gel mobility shift assays were carried out with sense and antisense RNA probes that contain both of the putative PREs found in the *pkaC* gene. Radiolabeled RNA probes were mixed with Ni^{+}-agarose partially purified protein extracts from the indicated strains during vegetative growth (veg) and the aggregation phase (agg). The arrow indicates the position of the shifted probe. (C) Competition with unlabeled sense and antisense probe. Partially purified wild-type cell extracts were incubated with radiolabeled sense $pkaC_{PRE}$ in the presence of unlabeled sense or antisense $pkaC_{PRE}$. Competition was performed with a 10-, 50- or 100-fold molar excess of unlabeled RNA.

complex was consistently observed with eluates made from *yakA*$^{-}$ cells (Fig. 6B). The binding of the sense probe to the complex could be effectively competed with unlabeled sense RNA, but not with anti-sense RNA (Fig. 6C). These experiments demonstrate the presence of factors in growing cells that bind specifically to the putative *pkaC* PREs. Furthermore, the formation of this complex appears to require a functional *pufA* gene. Comparing these results with the PKA-C protein measurements above leads to the observation that PRE complex formation correlates inversely with PKA-C protein levels. These results are consistent with a role for PufA in inhibiting the translation of *pkaC* mRNA through the PREs.

Expression of the putative *pkaC* PREs phenocopies a *pufA* mutation

A model where PufA binds to the *pkaC* PREs and inhibits *pkaC* mRNA translation predicts that expression of the *pkaC* PRE element would produce cells with a phenotype similar to *pufA*$^{-}$ cells. In this scenario, a high copy-number of PREs might titrate the available PufA protein, allowing for unregulated *pkaC* mRNA translation. Expression of the *pkaC* PREs in wild-type cells under the control of the *actin6* promoter did, in fact, lead to rapid aggregation and a slug phenotype similar to the *pufA*$^{-}$ cells (Fig. 7). However, these cells resumed culmination and formed fruiting bodies faster than *pufA*$^{-}$ cells, possibly because the *actin6* promoter used to drive PRE expression is not active after 16 hours of development in wild-type cells (Knecht et al., 1986). Expression of the *pkaC* PREs also resulted in increased PKA-C activity and protein levels (Table 1; Fig. 5B). The expression of the *pkaC* PREs in the antisense orientation produced no phenotype in wild-type cells (data not shown). These results show that expression of the *pkaC* PREs can disrupt the regulation of PKA-C translation and suggest that regulators that bind to this element control the initiation and timing of development in wild-type cells.

The repression of *PufA* expression requires *yakA*

To study the nature of the negative regulation of YakA upon PufA that was suggested from our genetic results, we analyzed

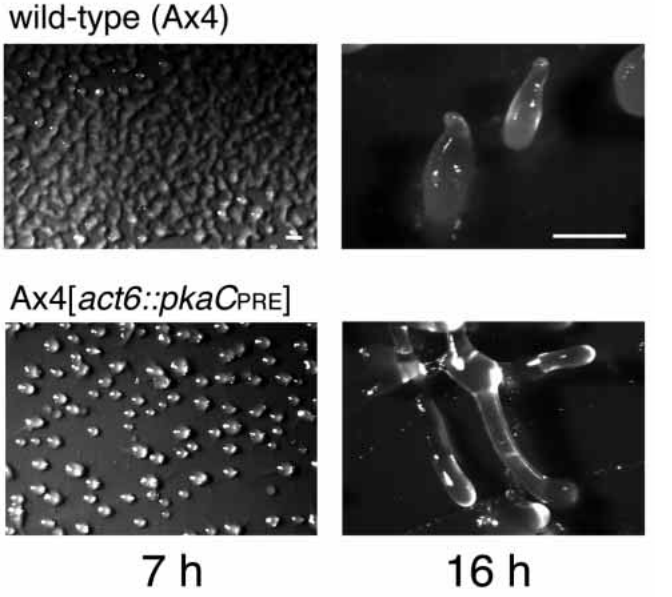

Fig. 7. Phenocopy of cells expressing the *pkaC* PREs. Bright-field microscopy of wild-type cells (Ax4), and Ax4 cells expressing the $act6::pkaC_{PRE}$ construct. Cells were grown in HL-5 and plated for development on filters for 7 and 16 hours. Bars; 0.5 mm.

pufA mRNA in starving wild-type and *yakA*$^-$ cells using an RNAse protection assay. As shown in Fig. 8, *pufA* mRNA was expressed during vegetative growth, decreased dramatically by 2 hours of development, was present at very low levels between 5 and 16 hours and then increased later at the culmination phase of development. Interestingly, we observed little, if any, decrease in *pufA* mRNA in *yakA*$^-$ cells (Fig. 8). This places *pufA* in the class of genes that includes *cprD*, whose decrease in expression at the onset of development requires YakA.

In an attempt to identify PufA, we carried out a partial purification of soluble proteins on nickel-agarose affinity columns, using extracts prepared from cells collected throughout development. Analysis of the resulting protein profile using an anti-5xHistidine antibody revealed one major band at an apparent molecular weight of 90 kDa, which is the predicted size for PufA (Fig. 8B). The 90 kDa protein also displayed an expression profile coincident with the PufA mRNA in that it decreased after cells were starved and it reappeared during culmination. In addition, this protein was not detected in *pufA*$^-$ cells, or in *yakA*$^-$ *pufA*$^-$ cells. Finally, the decrease in the 90 kDa protein at the onset of development is dependent on YakA, since the protein does not decrease as *yakA*$^-$ cells are starved (Fig. 8B). It is likely that this 90 kDa protein is PufA since it binds to nickel, it is of the expected size, it is missing in *pufA*$^-$ cells and its presence is coincident with PufA mRNA, both during the development of wild-type cells and in *yakA*$^-$ cells. These results suggest that YakA is required for the repression of PufA expression at the onset of development. Such a regulatory relationship could explain the ability of a *pufA* mutation to suppress the developmental deficiency of *yakA*$^-$ cells.

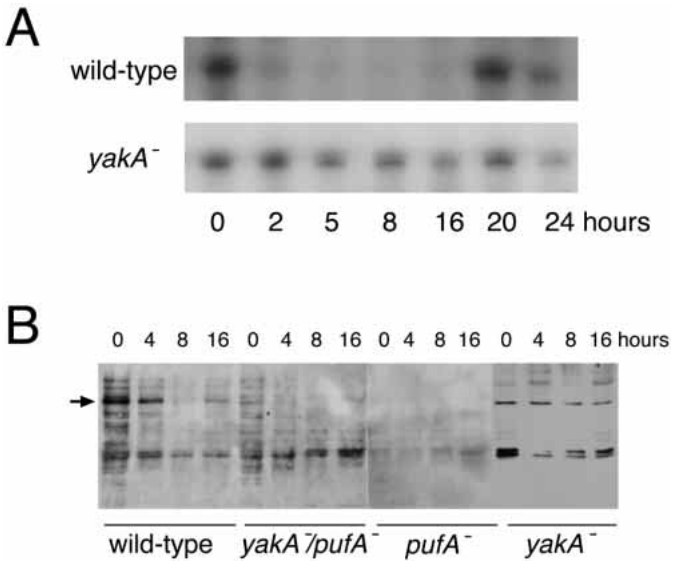

Fig. 8. Expression of PufA is regulated by YakA. (A) RNAse protection assays were used to determine the timing of *pufA* expression during growth and development. *pufA* mRNA levels in wild-type and in *yakA*$^-$ cells were determined from cells that were growing (t=0) or developing on filters for the indicated times. Control experiments demonstrated that the assays were carried out with >10-fold molar excess of probe (not shown). (B) Cell extracts were prepared from growing cells and developing cells of the strains indicated. Proteins that were absorbed and eluted from Ni^{2+}-agarose columns were detected by western blot analysis using anti-5xHis antibodies (see Materials and Methods). The arrow indicates the expected mobility of PufA.

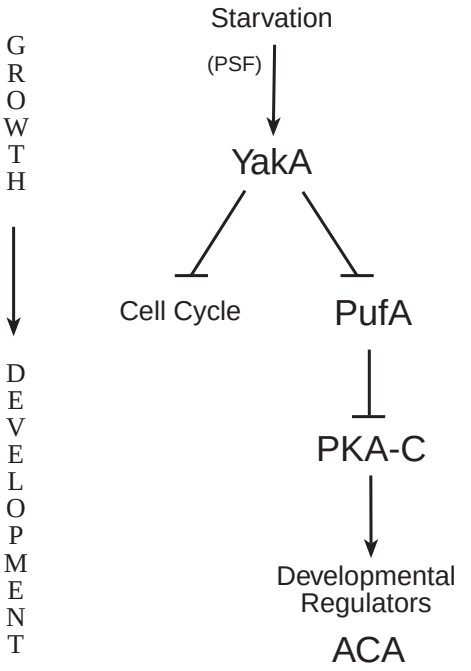

Fig. 9. A model of YakA and PufA function. Lines with bars indicate negative regulation while lines with arrowheads indicate positive regulation. The regulation of PKA-C by PufA is proposed to be a direct inhibition of translation. The inhibition of the 'cell cycle' indicates that YakA either positively regulates unknown cell cycle inhibitors, or negatively regulates cell cycle promoters, and is inferred from the phenotypes of *yakA*$^-$ and overexpressing cells. None of the other regulatory interactions are known to be direct but likely involve transcriptional regulation. See text for details.

DISCUSSION

The regulation of translation permits a cell to respond rapidly to changes in the environment, to accumulate mRNAs for later use, to form protein gradients and to control cell fate (reviewed by Curtis et al., 1995). RNA-binding proteins that recognize 3′ UTR elements in *Drosophila* control a cascade of events that will ultimately lead to the segmentation of the embryo. Examples include bicoid's regulation of translation of the transcription factor caudal (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996), bruno's regulation of oskar (Kim-Ha et al., 1995), smaug's regulation of nanos (Smibert et al., 1996) and pumilio's regulation of hunchback (Murata and Wharton, 1995). Pumilio, together with nanos, have been implicated in regulating the length of the poly(A) tail (Wreden et al., 1997). Using an indirect genetic assay, Wharton and co-workers (1998) have shown that pumilio can disrupt translation after the initiation step, suggesting that pumilio and nanos do not necessarily act by interfering with cap recognition. In *C. elegans*, sex determination is controlled by a cell-type switch orchestrated by the regulation of TRA-2 and FEM-3 translation. TRA-2 promotes egg formation while FEM-3 is required for sperm production (Goodwin et al., 1993; Ahringer

and Kimble, 1991), and recognition of the elements found in their 3′ UTR by RNA-binding proteins control the switch from sperm to oocyte production. The proteins that recognize the *fem-3* 3′ UTR element (PME), FBF-1 and FBF-2, appear to operate in an analogous way to pumilio, by inhibiting the translation of FEM-3.

A *Dictyostelium* Puf protein implicated in *pkaC* mRNA translational control

We have identified a new Puf protein family member, PufA, that regulates the production of the catalytic subunit of cAMP-dependent protein kinase, PKA-C. We have presented several pieces of evidence that suggest that PufA operates in the same manner as Pumilio and the FBFs, namely, by binding to the end of the *pkaC* mRNA and regulating its translation. First, our RNA-binding studies are consistent with the recognition and binding of PufA to the *pkaC* PREs. The PREs were defined based on the pattern of residues that have been shown to be critical for pumilio recognition of the NREs found in hunchback. Although the formation of a secondary 'stem-loop' structure has not been confirmed to occur in vivo, it is interesting to note that both the NREs and the putative PREs are predicted to form such structures (unpublished observations). The proteins that we observed to complex with the putative PREs bound to a nickel-agarose column, which is a property predicted for PufA. The complex was not detected in extracts derived from *pufA*$^-$ suggesting that PufA forms part of the complex, although it is formally possible that PufA is required for the production of PRE-binding proteins. Second, the 90 kDa PufA protein appears to be co-regulated with the level of the PRE-binding complex. Third, expression of the *pkaC* PREs gives a *pufA*$^-$ phenotype, consistent with the titration of PufA away from the PKA-C mRNA. Fourth, the amount of PKA-C activity and protein correlates inversely with the presence of PufA or the *pkaC* PRE complex, for all times of growth and development and in *pufA*$^-$, *pufA*$^-$ *yakA*$^-$, *yakA*$^-$ and *yakA* overexpression mutants. All of these results suggest that PufA binds to the PREs and regulates the translation of PKA-C directly.

YakA's 'dual' role in starvation sensing: growth arrest and induction of PKA-C

Our genetic analysis of *pufA* has clarified the two different functions of YakA. The *pufA* mutation rescues development, but clearly does not alter the distinctive growth properties of *yakA*$^-$ cells. The *yakA*$^-$ *pufA*$^-$ cells retain the small cell phenotype and rapid cell cycle of *yakA*$^-$ cells, whereas *pufA*$^-$ cells have no obvious cell cycle defects. PufA is normally produced during growth and its levels decline at the onset of development, but PufA does not appear to be required for vegetative growth in that we could detect no gross differences between wild-type cells and *pufA*$^-$ cells in terms of growth rate or cell appearance. However, PufA is required to prevent the expression of developmental genes such as *acaA*, so in this sense PufA has a role in maintaining the growth state. The decrease in vegetative gene expression that normally occurs in the first few hours of development is also dependent on YakA but not on PufA. The *yakA*$^-$ *pufA*$^-$ cells express *cprD* for many hours during the first half of development, but *pufA*$^-$ cells decrease *cprD* expression as wild-type cells do.

These results suggest a model in which YakA regulates the cell cycle during growth, whereas the induction or activation of YakA upon starvation leads to a decrease in vegetative gene expression with the consequent relief of the PufA-mediated translational block on *pkaC* mRNA (Fig. 9). These two aspects of YakA function may be mediated by distinct pathways, but it is also possible that the repression of vegetative gene expression is an indirect by-product of cell cycle arrest. Conversely, cell cycle arrest may be accomplished by YakA via the repression of essential growth genes through the same pathway that regulates *pufA*. In addition, other regulatory pathways that are independent of YakA must mediate cell cycle arrest since *yakA*$^-$ cells do arrest growth in response to starvation (Souza et al., 1998). However, these other pathways clearly do not induce development in the absence of YakA. Our interpretation of YakA's role in cell cycle arrest comes from the observation that overexpression of this kinase induces cell cycle arrest without cell death, and from its role in the regulation of the interval in between cell divisions (Souza et al., 1998). The induction of PKA-C is not likely to be a critical component of YakA-mediated cell cycle arrest since we have been unable to discern any effect of PKA-C overexpression on growth (unpublished observations). Although the role of YakA in cell cycle arrest is not as yet clear, our genetic observations do indicate that PufA is a key effector of that part of the YakA starvation response pathway that leads to multicellular development.

Induction of PKA-C and the initiation of development

The proper regulation of PKA-C activity is essential for almost every aspect of *Dictyostelium* development and there is substantial evidence that an increase in the synthesis of PKA-C is essential for the initiation of development (recently reviewed by Loomis, 1998). PKA-C mRNA, protein and activity all increase about 5-fold in the first 4-6 hours of development (Leichtling et al., 1984; Mann and Firtel, 1992; Anjard et al., 1993), and numerous genetic manipulations have suggested that this is a required event (Firtel and Chapman 1990; Mann and Firtel, 1991; Anjard et al. 1992; Harwood et al., 1992; Mann et al., 1997; Souza et al., 1998). The aggregation-deficient phenotype of *pkaC*$^-$ cells show that PKA-C is required for the initiation of development. This is confirmed by the lack of early developmental gene expression in these cells that would normally occur in the first few hours after starvation (Schulkes and Schaap, 1995; Mann et al., 1997). Our work with YakA illustrates the point that a 5-fold increase in PKA-C protein is sufficient to induce development under starvation conditions. The vegetative levels of PKA-C present in *yakA*$^-$ cells are not sufficient to induce ACA expression or development, but the expression of about 5-times more PKA-C appears to completely bypass the requirement for YakA and rescues development in these cells. Inactivation of *pufA* rescues the early phases of development of *yakA*$^-$ cells, but completion of fruiting body formation seems to be compromised since we observe an arrest at the culmination phase. *yakA*$^-$ cells that overexpress *pkaC* exhibit faster development without culmination aberrations, which may reflect the existence of additional functions for the PufA protein during the later phases of development. PKA-C controls the timing of early developmental events through its regulation of the expression of key cAMP signaling proteins

such as cAR1 and ACA, probably mediated by transcription factors such as Myb2 (Anjard et al., 1992; Mann et al., 1992, 1997; Otsuka and van Haastert, 1998). Regulating the translation of PKA-C may cause an imbalance with the PKA regulatory subunit, PKA-R, possibly allowing PKA-mediated events independent of cAMP signaling. Imbalances in the PKA-C/PKA-R ratio have been suggested to promote later functions of PKA that appear to be independent of intracellular cAMP (Mann and Firtel, 1993). The potential for a transient excess of PKA-C might explain how PufA translational regulation could exert sensitive control over the initiation of development.

Our studies emphasize the position of YakA as a critical node in the regulatory network that governs the growth to development transition in *Dictyostelium*. In budding yeast, Yak1p regulates the response to thermal stress that results in heat resistance. The similarities between YakA and Yak1p points to a functional conservation of Yak kinases as general regulators of stress responses that may extend to other systems. Of particular interest are the Yak-related minibrain kinases that control the growth and/or differentiation of neuroblasts in mammals and *Drosophila* (Tejedor et al., 1995; Smith et al., 1997). Our studies also suggest that Puf-mediated translational control evolved prior to the divergence of metazoan species. It will be of interest to determine whether Puf proteins are effectors for Yak/minibrain kinases in the growth to development decisions of other eukaryotes.

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REFERENCES

Adachi, H., Hasebe, T., Yoshinaga, K., Ohta, T. and Sutoh, K. (1994). Isolation of *Dictyostelium discoideum* cytokinesis mutants by restriction enzyme-mediated integration of the blasticidin S resistance marker. *Biochem. Biophys. Res. Comm.* **205,** 1808-1814.

Ahringer, J. and Kimble, J. (1991). Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem-3* 3′ untranslated region. *Nature* **349,** 346-348.

Anjard, C., Pinaud, S., Kay, R. R. and Reymond, C. D. (1992). Over-expression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*. *Development* **115,** 785-790.

Anjard, C., Etchebehere, L., Pinaud, S., Veron, M., and Reymond, C. D. (1993). An unusual catalytic subunit for the cAMP-dependent protein kinase of *Dictyostelium discoideum. Biochemistry* **32,** 9532-9538.

Burki, E., Anjard, C., Scholder, J. C. and Reymond, C. D. (1991). Isolation of two genes encoding putative protein kinases regulated during *Dictyostelium discoideum* development. *Gene* **102,** 57-65.

Clarke, M. and Gomer, R. H. (1995). PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*. *Experientia* **51,** 1124-1134.

Curtis, D., Lehmann, R. and Zamore, P. D. (1995). Translational regulation in development. *Cell* **81,** 171-178.

da Silva, A. M. and Klein, C. (1990). Cell adhesion in transformed *Dictyostelium discoideum* cells: expression of gp80 and its biochemical characterization. *Dev. Biol.* **140,** 139-148.

Dubnau, J. and Struhl, G. (1996). RNA recognition and translational regulation by a homeodomain protein. *Nature* **379,** 694-699.

Early, A. E. and Williams, J. G. (1988). A *Dictyostelium* prespore-specific gene is transcriptionally repressed by DIF in vitro. *Development* **103,** 519-524.

Firtel, R. A. (1996). Interacting signaling pathways controlling multicellular development in *Dictyostelium. Curr. Opin. Genet. Devel.* **6,** 545-54

Firtel, R. A. and Chapman, A. L. (1990). Role for cAMP-dependent protein kinase-A in early *Dictyostelium* development. *Genes Dev.* **4**, 18-28.

Garrett, S. and Broach, J. (1989). Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, *YAKI*, whose product may act downstream of the cAMP-dependent protein kinase. *Genes Dev.* **3**, 1336-1348.

Garrett, S., Menold, M. M. and Broach, J. R. (1991). The *Saccharomyces cerevisiae YAK1* gene encodes a protein kinase that is induced by arrest early in the cell cycle. *Mol. Cell. Biol.* **11**, 4045-4052.

Goodwin, E. B., Okkema, P. G., Evans, T. C. and Kimble, J. (1993). Translational regulation of *tra-2* by its 3′ untranslated region controls sexual identity in *C. elegans. Cell* **75,** 329-339.

Harlow, E. and Lane, D. (1988). *Antibodies, a Laboratory Manual*. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Hartley, A. D., Ward, M. P. and Garrett, S. (1994). The Yak1 protein kinase of *Saccharomyces cerevisiae* moderates thermotolerance and inhibits growth by an Sch9 protein kinase-independent mechanism. *Genetics* **136,** 465-474.

Harwood, A. J., Hopper, N. A., Simon, M.-N., Driscoll, D. M., Veron, M. and Williams, J. G. (1992). Culmination in *Dictyostelium* is regulated by the cAMP-dependent protein kinase. *Cell* **69,** 615-624.

Jang, A. and Hill, R. P. (1997). An examination of the effects of hypoxia, acidosis, and glucose starvation on the expression of metastasis-associated genes in murine tumor cells. *Clin. Exp. Metastasis* **15,** 469-83.

Kim-Ha, J., Kerr, K. and MacDonald, P. M. (1995). Translational regulation of *oskar* mRNA by Bruno, an ovarian RNA-binding protein, is essential. *Cell* **81,** 403-412.

Knecht, D. A., Cohen, S. M., Loomis, W. F. and Lodish, H. F. (1986). Developmental regulation of *Dictyostelium* discoideum actin gene fusions carried on low-copy and high-copy transformation vectors. *Mol. Cell. Biol.* **6,** 3973-3983.

Kuspa, A. and Loomis, W. F. (1992). Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci., USA* **89,** 8803-8807.

Kuspa, A. and Loomis, W. F. (1994). Transformation of *Dictyostelium* – gene disruptions, insertional mutagenesis, and promoter traps. *Meth. Mol. Genet.* **3,** 3-21.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

Leichtling, B. H., Majerfeld, I. H., Spitz, E., Schaller, K. L., Woffendin, C., Kakinuma, S. and Rickenberg, H. V. (1984). A cytosolic cyclic AMP-dependent protein kinase in *Dictyostelium discoideum*. II. Developmental regulation. *J. Biol. Chem.* **259,** 662-668.

Loomis, W. F. (1998). Role of PKA in the timing of developmental events in *Dictyostelium. Microbiol. Mol. Biol. Rev.* **62,** 684-694.

MacDonald, P. M. (1992). The *Drosophila pumilio* gene: and unusually long transcription unit and an unusual protein. *Development* **114,** 221-232.

Maeda, Y. (1997). Cellular and molecular mechanisms of the transition from growth to differentiation in *Dictyostelium* cells. In *Dictyostelium – A Model System for Cell and Developmental Biology*, pp. 207-218. Universal Academy Press, Tokyo, Japan.

Mann, S. K. O. and Firtel, R. A. (1991). A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*. *Mech. Devel*. **35,** 89-102.

Mann, S. K. O. and Firtel, R. A. (1993). cAMP-dependent protein kinase differentially regulates prestalk and prespore differentiation during *Dictyostelium* development. *Development* **119,** 135-146.

Mann, S. K. O., Yonemoto, W. M. Taylor, S. and Firtel, R. A. (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.

Mann, S. K. O, Brown, J. M., Briscoe, C., Parent, C., Pitt, G., Devreotes, P. and Firtel, R. A. (1997). Role of cAMP-dependent protein kinase in controlling aggregation and post-aggregative development in *Dictyostelium. Dev. Biol*. **183,** 208-221.

Murata Y. and Wharton, R. P. (1995). Binding of Pumilio to maternal hunchback mRNA is required for posterior patterning in Drosophila embryos. *Cell* **80,** 747-756.

Nellen, W., Datta, S., Reymond, C., Sivertsen, A., Mann, S., Crowley, T. and Firtel, R. A. (1987). Molecular biology in *Dictyostelium*: tools and applications. *Meth. Cell Biol.* **28**: 67-100.

Ohmori, R. and Maeda, Y. (1987). The developmental fate of *Dictyostelium discoideum* cells depends greatly on the cell cycle position at the onset of starvation. *Cell Differ.* **22,** 11-18.

Otsuka, H. and Van Haastert, P. J. (1998). A Myb homolog initiates *Dictyostelium* development by induction of adenylyl cyclase expression. *Genes Dev.* **12,** 1738-1748.

Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R. and Devreotes, P. N. (1992). Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell* **69,** 305-315.

Rathi, A., Kayman, S. C. and Clarke, M. (1991). Induction of gene expression in *Dictyostelium* by Pre-Starvation Factor, a factor secreted by growing cells. *Dev. Genet.* **12,** 82-87.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J. and Jackle, H. (1996). RNA binding and translational suppression by bicoid. *Nature* **379,** 746-749.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schulkes, C. and Schaap, P. (1995). cAMP-dependent protein kinase activity is essential for pre-aggregative gene expression in *Dictyostelium*. *FEBS Lett.* **368,** 381-384.

Simon, M.-N., Pelegrini, O., Veron, M. and Kay, R. R. (1992). Mutation of protein kinase A causes heterochronic development of *Dictyostelium. Nature* **356,** 171-172.

Smibert, C. A., Wilson, J. E., Kerr, K. and Macdonald, P. M. (1996). smaug protein represses translation of unlocalized nanos mRNA in the *Drosophila* embryo. *Genes Dev.* **10,** 2600-2609.

Smith, A., Ward, M. P. and Garrett, S. (1998). Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. *EMBO J.* **17**, 3556-3564.

Smith, D. J., Stevens, M. E., Sudanagunta, S. P., Bronson, R. T., Makhinson, M., Watabe, A. M., O'Dell, T. J., Fung, J., Weier, H.-U. G., Cheng, J.-F. and Rubin, E. M. (1997). Functional screening of 2 Mb of human chromosome 21q22.2 in transgenic mice implicates minibrain in learning defects associated with Down syndrome. *Nat. Genetics* **16,** 28-36.

Souza, G. M., Hirai, J. and Freeze, H. H. (1995). Identification of two novel *Dictyostelium discoideum* cysteine proteinases that carry N-acetylglucosamine-1-P modification. *J. Biol. Chem.* **270,** 28938-28945.

Souza, G. M., Lu, S. and Kuspa, A. (1998). YakA, a protein kinase required for the transition from growth to development in *Dictyostelium*. *Development* **125,** 2291-2302.

Sussman, M. (1987). Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell. Biol.* **28,** 9-29.

Tejedor, F., Zhu, X. R., Kaltenbach, E., Ackermann, A., Baumann, A., Canal, I., Heisenberg, M., Fischbach, K. F. and Pongs, O. (1995). Minibrain- a new protein kinase family involved in postembryonic neurogenesis in Drosophila. *Neuron* **14,** 287-301.

Thevelein, J. M. (1994). Signal transduction in Yeast. *Yeast* **10,** 1753-1790.

Wang, B., Shaulsky, G. and Kuspa, A. (1999). Multiple developmental roles for CRAC, a cytosolic regulator of Adenylyl cyclase. *Dev. Biol.* **208,** 1-13.

Wharton, R. P., Sonoda, J., Lee, T., Patterson, M. and Murata, Y. (1998). The pumilio RNA-binding domain is also a translational regulator. *Molecular Cell* **1,** 863-872.

Wreden, C., Verrotti, A. C., Schisa, J. A., Lieberfarb, M. E. and Strickland, S. (1997). Nanos and pumilio establish embryonic polarity in *Drosophila* by promoting posterior deadenylation of hunchback mRNA. *Development* **124,** 3015-3023**.**

Yamamoto, M. (1996). Regulation of meiosis in fission yeast. *Cell Struct. and Func.* **21,** 431-6.

Zamore, P. D., Williamson, J. R. and Lehmann, R. (1997). The pumilio protein binds RNA through a conserved domain that defines a new class of RNA-binding proteins. *RNA* **3,** 1421-1433.

Zamore, P. D., Bartel, D. P., Lehmann, R. and Williamson, J. R. (1999). The PUMILIO-RNA interaction: a single RNA-binding domain monomer recognizes a bipartite target sequence. *Biochemistry* **38,** 596-604.

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J. and Wickens, M. P. (1997). A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390,** 477-484.