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Calcium levels correlate with cell cycle phase and affect the level of the cyclin B transcript in *Dictyostelium discoideum*

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Abstract

In pre-aggregation amoebae of *Dictyostelium discoideum*, phenotypic differences with respect to cellular Ca^{2+} and cell cycle phases are known to bias post-aggregative cell-type choice. Using chlortetracycline fluorescence as an indicator, we found that cellular Ca^{2+} is highest at the S phase of the cell cycle. Upon increasing the level of Ca^{2+} with the help of the calcium ionophore A23187, there is a significant decrease in the cyclin B (*clb1*) mRNA level; the *cdc2* mRNA level shows a marginal decrease. These results suggest that the effect of Ca^{2+} and the cell cycle on cell fate could be exerted at the level of transcription, or message stability, of specific genes. © 1998 Published by Elsevier Science B.V.

Keywords: Cell cycle; Calcium; Cyclin B; *Dictyostelium*

1. Introduction

In the social amoeba *Dictyostelium discoideum* the terminally differentiated structure consists of spores and a stalk of dead cells. Prior to this, the multicellular migrating slug that is formed by the aggregation of starved amoebae contains prestalk (or presumptive stalk) cells in approximately the anterior 20% and prespore (or presumptive spore) cells in the posterior 80% [1]. Levels of cytoplasmic and sequestered Ca^{2+} in vegetative amoebae are coordinately regulated [2]; high levels of both go with a prestalk tendency, whereas low levels indicate a prespore tendency [2–4]. Also, amoebae that are in the S or early (mid to late) G2 phase of the cell cycle at the time of starvation exhibit a prestalk (prespore) tendency [5,6]. Whether Ca^{2+} and the cell cycle act in series or parallel, and how one might affect the other, are not known. Ca^{2+} and divalent cations can act as inducers of gene expression and cell differentiation in *D. discoideum* and other systems [4,7–9]. In *D. discoideum*, Ca^{2+} levels differ between prestalk and prespore cells in the slug [4]. Further, artificially increasing (decreasing) cellular Ca^{2+} with the help of calcium ionophore A23187 and Ca^{2+}-EGTA buffers generates a stalky (spory) phenotype [9]. The molecular correlates of these striking effects

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on differentiation and morphogenesis remain unknown.

Control systems involved in the G2 to M phase transition of the cell cycle are highly conserved in eukaryotic cells. Initiation of mitosis requires a protein kinase complex consisting of catalytic (*cdc2* protein kinase) and regulatory (cyclin B) subunits. Cyclin B accumulates during G2 and reaches a maximum at the G2/M boundary. It is then destroyed during mitosis, causing the inactivation of *cdc2* protein kinase and exit from the M phase [10]. mRNA levels of the cyclin B homologue of *D. discoideum* (*clb1*) and those of the *clb1* protein oscillate during the cell cycle with maximum accumulation prior to cell division and maximum levels of protein occurring during cell division [11]. Cdc2 protein levels are constant during the inter-mitotic interval [12]. mRNA levels of *clb1* and *cdc2* are coordinately regulated during the synchronous growth of vegetative *D. discoideum* cells [12].

There are grounds for believing that Ca^{2+} can interact with the cell cycle. Through the interplay of Ca^{2+} and Ca^{2+} binding proteins, cells regulate key steps in the cell cycle such as reentry of quiescent cells into the proliferation state and transitions through the G1/S, G2/M and the metaphase/anaphase boundaries [13]. In rat fibroblasts, the levels of Ca^{2+} in different cell cycle phases have been shown to vary cyclically [14]. These results motivated us to look for a link between calcium and cell cycle-specific gene expression in *D. discoideum*.

2. Materials and methods

2.1. Growth and development of cells

D. discoideum (Ax-2) amoebae were grown in HL-5 medium [15]. Amoebae were harvested by centrifugation at $400 \times g$ for 5 min, washed with MES (2-[*N*-morpholino]ethanesulfonic acid buffer; pH 6.4), resuspended in the same buffer and either kept shaking at 10^7 cells ml^{-1}, or plated for development on 2% non-nutrient agar, both at 22°C. All chemicals

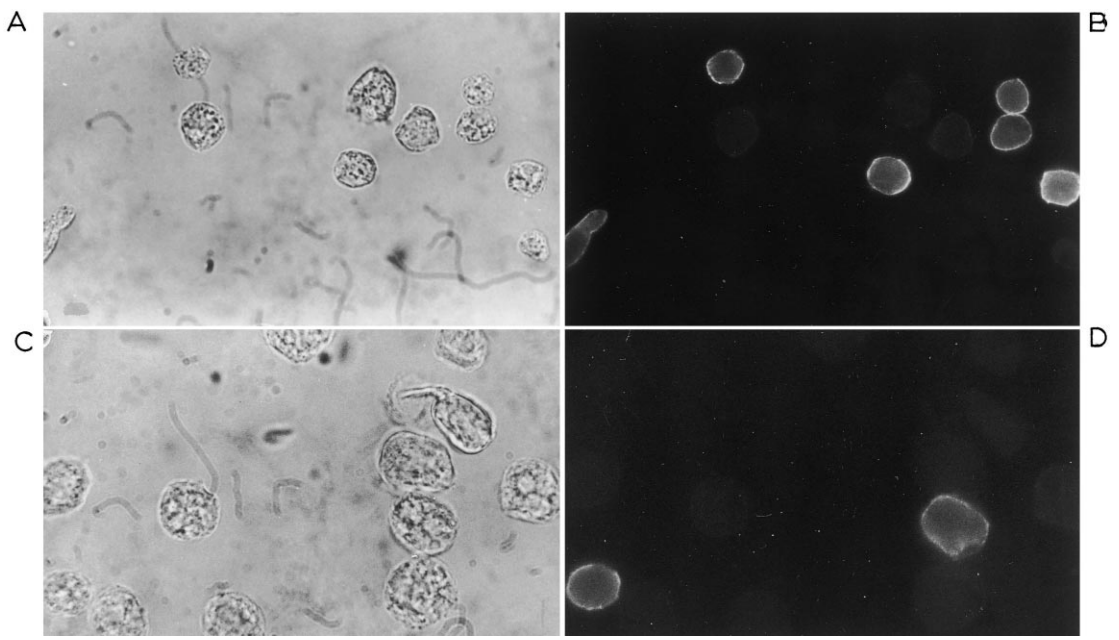

Fig. 1. Bright field (A,C) and corresponding fluorescence (B,D) images of synchronous Ax-2 cells depicting MUD 9 binding to the cell surface. A,B: 4 h after release from stationary phase. C,D: 7 h after release. Cells were fixed, processed and incubated with MUD 9-FITC monoclonal antibody (10 μg ml^{-1}) as described. Note the higher fraction of cells displaying MUD 9 binding in A,B. Magnification: ×205 (A,B); ×290 (C,D).

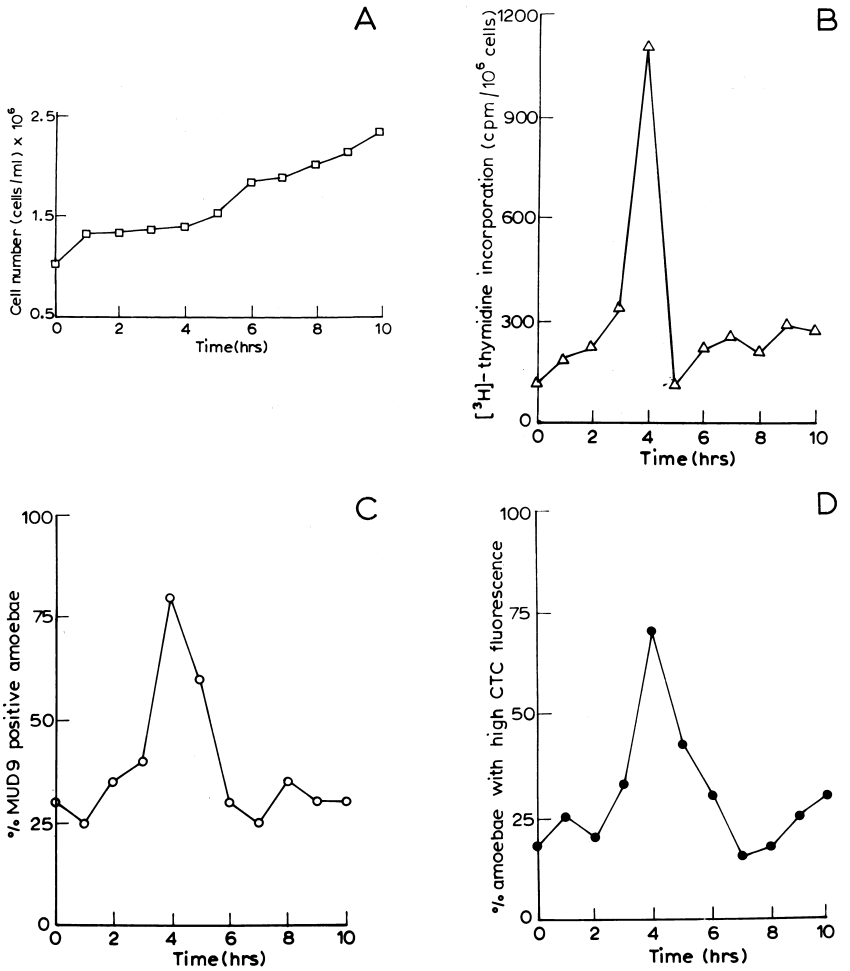

Fig. 2. A representative experiment showing (A) relative increase in cell density, (B) incorporation of [^{3}H]thymidine into pulse-labelled cells (cpm per 10^6 cells), (C) percentage of cells with MUD 9 binding and (D) percentage of cells displaying high sequestered Ca^{2+} (all data from Ax-2 cells after release from stationary phase). Note that about 4 h after release from stationary phase, cells show a peak of [^{3}H]thymidine incorporation, exhibit high sequestered Ca^{2+} and display maximum MUD 9 binding.

were of analytical grade and obtained from either Merck or Sigma Chemicals (USA). A23187 was obtained from Molecular Probes Inc., USA.

2.2. *Monitoring of cell cycle phases and cellular Ca^{2+} in synchronous cultures*

Cells were synchronized by dilution of stationary phase cells into fresh medium as described by Weijer et al. [5] and the cell density monitored by direct counting. Aliquots were removed hourly and three portions were separately analyzed. DNA synthesis was followed by pulse-labelling of cells with [^{3}H]thymidine (Jonaki, Hyderabad, India; specific activity 80–90 Ci $mmol^{-1}$). [^{3}H]Thymidine was added to 3 ml of cells at a final concentration of 25 μCi ml^{-1} and cells were incubated with shaking for 30 min at 22°C. Cells were processed and radioactivity was determined using a Packard 3000 scintillation counter as in [5]. MUD 9 monoclonal antibody coupled to fluorescein-isothiocyanate (MUD 9-FITC) was used to independently score cells that were in S phase [16]. Other cells were fixed with 4% paraformaldehyde buffered in PBS (0.15 M

NaCl, 0.01 M Na,K-phosphate, pH 7.4) and processed for MUD 9-FITC staining [16]. About 50 cells from each time point were observed and scored for MUD 9-FITC fluorescence under a Leitz MPV3 fluorescence microscope. Finally, some cells from each fraction were also incubated for 30 min at 22°C with 80 μM chlortetracycline (CTC), a prestalk marker and a fluorescent probe of sequestered Ca^{2+} [17].

2.3. Perturbation of cellular Ca^{2+}

Cellular Ca^{2+} levels of asynchronous and axenically growing amoebae of *D. discoideum* were artificially increased by the use of the calcium ionophore A23187 (7 μM) and $CaCl_2$ (1 mM) as described [9]. Similarly, Ca^{2+} levels were decreased by the use of A23187 (7 μM) and EGTA (1 mM). In both cases the amoebae were shaken in HL-5 at 180 rpm for 2 h at 22°C. After this they were washed with 10 mM MES, centrifuged, and the pellet was used for the isolation of total RNA.

2.4. Slot blot analysis

RNA was extracted from vegetative amoebae by the acid guanidinium thiocyanate method [18] as follows. RNA from control cells (untreated), cells treated with A23187 alone, EGTA alone, A23187+EGTA, $CaCl_2$ alone, and A23187+$CaCl_2$ was selectively precipitated by the addition of an equal volume of ice-cold 5 M LiCl/ethanol (3/2 v/v) and the mixture was stored overnight at 4°C to allow complete precipitation. The supernatant was removed after centrifugation for 15 min in a microfuge and the pellet washed several times with 70% ethanol to remove traces of LiCl. RNA was treated with RNase-free DNase (Sigma Chemicals, USA) in the presence of RNase inhibitor to remove contaminating DNA. RNA was re-extracted and precipitated again with 100% ethanol at −20°C for 2–3 h. RNA was pelleted in a microfuge, washed several times with 70% ethanol, air-dried and dissolved in DEPC-treated water. RNA quantification was carried out spectrophotometrically. For slot blot analysis filtration manifolds (Minifold II, Schleicher and Schuell) were used. 10 μg RNA of each sample was used on the blot. Blotting was carried out following the protocol of Sambrook et al. [19]. RNA was cross-linked to the nylon membrane by UV irradiation for 5 min.

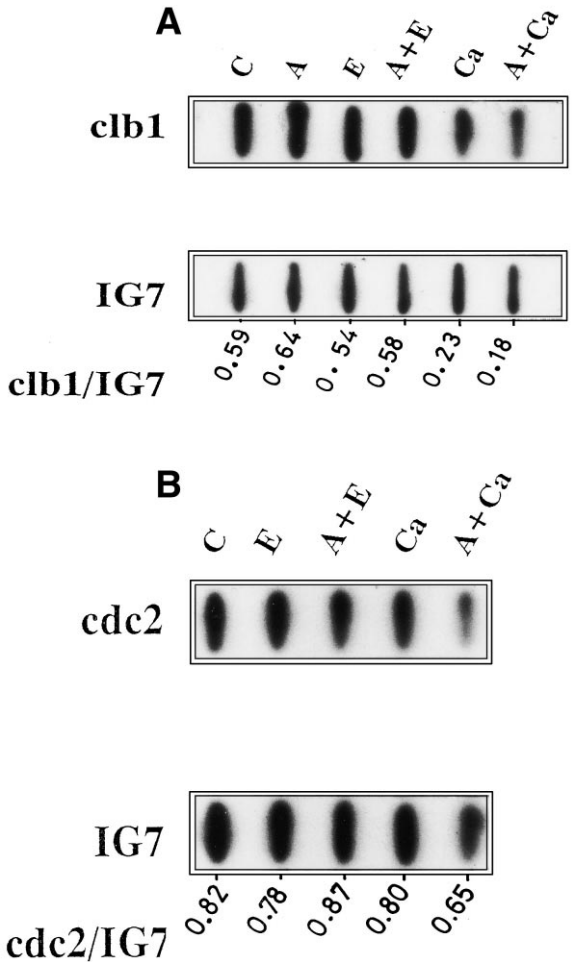

Fig. 3. A, B: Slot blot analysis of RNA isolated under different experimental conditions. Total RNA was extracted from cells treated for 2 h as follows: control (untreated; lane labelled C); 7 μM A23187 alone (A); 1 mM EGTA alone (E); 7 μM A23187+1 mM EGTA (A+E); 1 mM Ca^{2+} alone (Ca); and 7 μM A23187+1 mM Ca^{2+} (A+Ca). Corresponding to each set of conditions, total RNA (10 μg) was transferred to a nylon membrane and probed with a 514-bp *Bgl*II/*Eco*RI *clb1* cDNA fragment (A, top panel) or a 1-kb *Eco*RI *cdc2* fragment (B, top panel). In both panels the blots at the bottom show the results of probing with a 500-bp IG7 control fragment. The *clb1* (or *cdc2*) probe was stripped from the blot by treatment for 20 min at 65°C with 0.075% SDS, 0.075×SSC, 25% formamide and the blots were reprobed with the IG7 DNA. *clb1* to IG7 (or *cdc2* to IG7) transcript ratios, shown as fractions below the lanes, were estimated by scanning densitometry of the autoradiographs.

2.5. Hybridization

For hybridization a 1.0-kb *Eco*RI fragment of the *cdc2* cDNA and a 514-bp *Bgl*II/*Eco*RI fragment of the *clb1* cDNA were used as probes [20,11]. The probes were labelled by the random oligonucleotide primer method by using [$\propto$-^{32}P]dATP [21]. For hybridization with the cyclin B cDNA probe, nylon membranes were prehybridized for 2–3 h and then hybridized overnight at 42°C in a solution containing 50% formamide, 5×SSC, 5×Denhardt's, 0.5% SDS and 250 μg ml^{-1} of sheared and denatured salmon sperm DNA. For hybridization with *clb1*, a denatured radio-labelled probe was added to the hybridization mix. After hybridization the blot was washed twice for 15 min in 0.5×SSC, 0.1% SDS at 60°C and exposed to X-ray film. For *cdc2* hybridization, the membranes were prehybridized for 2–3 h in 15×SSC, 1% SDS and 0.5% dried milk powder at 60°C. Prehybridized membranes were later probed with the addition of a radio-labelled denatured cDNA probe of *cdc2*. Hybridization was carried out for 16 h at 60°C. The blot was then washed at 60°C for 30 min with 0.1×SSC, 0.1% SDS and exposed to X-ray film. IG7 DNA (kindly donated by J. Gross) was used as an internal standard in order to normalize the mRNA levels in different experiments [22]. The IG7 fragment was radio-labelled, hybridized and washed in a same way as cyclin B (*clb1*). The autoradiographs were scanned with a densitometer and the ratios of *cdc2* to IG7 and *clb1* to IG7 transcript were compared under different conditions. The data were analyzed statistically using Student's *t*-test.

3. Results

3.1. Calcium levels vary with cell cycle phase

Aliquots of synchronized amoebae were removed at regular intervals and monitored for [^{3}H]thymidine incorporation into DNA, CTC fluorescence and the presence of antigen specific to the MUD 9 monoclonal antibody. Three independent experiments showed that the fraction of cells showing a high level of CTC fluorescence, as well as the fraction of cells that bound MUD 9, peaked at the same phase of the cell cycle as that showing maximal [^{3}H]thymidine incorporation (Figs. 1A,B and 2A–D).

3.2. High levels of cellular Ca^{2+} significantly reduce the cyclin B (clb1) message

Densitometric scanning of slot blots showed that there was a significant decrease, amounting to 2–3-fold, in the level of *clb1* mRNA when growing amoebae of *D. discoideum* were exposed to 7 μM A23187 and 1 mM Ca^{2+} for 2 h (compared to untreated amoebae; see lanes 1 and 6 in Fig. 3A; Table 1). Under conditions that decreased Ca^{2+}, the level of *clb1* mRNA did not display any significant change (see Fig. 3A, lanes 1, 3 and 4). High levels of extracellular Ca^{2+} (1 mM) alone led to a slight decrease in the *clb1* mRNA level but this was statistically insignificant (compare lanes 1 and 5 and lanes 5 and 6, Fig. 3A; Table 1). By itself, neither EGTA (1 mM) nor A23187 (7 μM) caused any significant change in the *clb1* mRNA level (compare lanes 1 and 3 in Fig. 3A; Table 1). The level of expression of IG7, a constitutive gene, was not affected by any of the treatments (see IG7 panel of Fig. 3A).

Table 1
Effect of cellular Ca^{2+} on cyclin (*clb1*) and *cdc2* mRNA levels

Experimental conditions	Relative transcriptional level of cyclin B (*clb1*)	Relative transcriptional level of *cdc2*
Control	0.34 ± 0.17	0.51 ± 0.21
+7 μM A23187	0.41 ± 0.22	0.32 ± 0.14
+1 mM EGTA	0.17 ± 0.05*	0.45 ± 0.24
+1 mM Ca^{2+}	0.17 ± 0.05*	0.40 ± 0.28
+7 μM A23187+1 mM EGTA	0.30 ± 0.19	0.44 ± 0.30
+7 μM A23187+1 mM Ca^{2+}	0.11 ± 0.04**	0.31 ± 0.23

Relative levels of transcription are shown as the ratio of *clb1*/IG7 and *cdc2*/IG7 in each condition. Values represent mean ± S.D. (n = 3).
*Statistically insignificant; **statistically significant at the 5% level.

3.3. The cdc2 mRNA level falls marginally under high cellular Ca^{2+} conditions

When Ca^{2+} was raised by the use of A23187, a decrease of 1.5-fold in the level of the *cdc2* transcription was seen. This was not statistically significant at the 5% level (see lanes 1 and 5 in Fig. 3B; Table 1). Low cellular Ca^{2+} did not have any significant effect on the *cdc2* mRNA at the 5% level (see Table 1). Similarly, there was no significant change in the *cdc2* mRNA level with high extracellular Ca^{2+} (1 mM), EGTA (1 mM) (compare Fig. 3B, lanes 1 and 4, also lanes 1 and 2; Table 1) and A23187 (7 μM) (see Table 1).

4. Discussion

4.1. Amoebae in S phase contain relatively high levels of Ca^{2+}

CTC fluorescence is a direct indicator of sequestered Ca^{2+} inside the cell [17]. Because levels of free and sequestered Ca^{2+} are correlated in freshly starved amoebae [2], indirectly it is also an indicator of free or cytoplasmic Ca^{2+}. The MUD 9 monoclonal antibody binds specifically to cells in the S phase of the cell cycle [16]. [^{3}H]Thymidine uptake, which monitors DNA synthesis, should also peak at the S phase, as indeed it does (compare Fig. 1A,B with C,D). The reason for using the antibody is to make sure that the picture is not complicated by [^{3}H]thymidine incorporation into mitochondrial DNA (which comprises up to 50% of total DNA in *D. discoideum*; [5] and unpublished results). Basically, what Fig. 2 shows is that cellular Ca^{2+} – sequestered or free – exhibits a significant peak at the S phase of the cell cycle. Though we have not quantified Ca^{2+} levels at various phases, the observations discussed below suggest that this natural peak affects the transcript level of at least one 'cell cycle gene'.

4.2. Ca^{2+} affects the level of the cyclin B message

Cellular Ca^{2+} levels have been perturbed with the help of EGTA or Ca^{2+} in the extracellular medium along with A23187, which equilibrates cellular Ca^{2+} with that of extracellular Ca^{2+} [9]. Control experiments ruled out the possibility that A23187 had any effect on cell viability or cell proliferation (not shown). Transcript levels were estimated after normalizing the cyclin B and *cdc2* message levels with that of the constitutively expressed IG7 gene [22]. The central observation is that upon increasing the level of cellular Ca^{2+} by the use of A23187, there is a 2–3-fold decrease ($P < 0.05$) in cyclin B (*clb1*) mRNA (Fig. 3A; Table 1). Without the ionophore, a high level of extracellular Ca^{2+} (1 mM) causes a slight, statistically insignificant ($P > 0.05$) decrease in the *clb1* mRNA level (Fig. 3A; Table 1). Schlatterer and Schaloske [23] found that high levels of extracellular Ca^{2+} led to a small increase in the cellular Ca^{2+}. Therefore, this slight decrease in *clb1* mRNA under high extracellular Ca^{2+} conditions is probably due to a correspondingly slight rise in cellular Ca^{2+}. An elevation of Ca^{2+} leads to a marginal decrease (1.5-fold on average) in the level of the *cdc2* transcript but this too is not significant ($P > 0.05$; Fig. 3B; Table 1). A decrease in cellular Ca^{2+} does not appear to influence either the *clb1* or the *cdc2* mRNA levels ($P > 0.05$; see Table 1). Artificially increasing (decreasing) cellular Ca^{2+} by A23187 and Ca^{2+}-EGTA buffers has been shown to enhance (reduce) the proportion of prestalk cells in the slug and generate a stalky (spory) phenotype in *D. discoideum* [9].

cdc2 and *clb1* mRNA levels are coordinately regulated during the synchronous growth of *D. discoideum* and are higher prior to cell division and in the early G2 phase of the cell cycle [12]. The mechanism by which Ca^{2+} might regulate the transcription of *clb1*, or the stability of the *clb1* message, is not known. A decrease in the level of the *clb1* product could block cells from progressing through the G2/M transition. If so, such cells would tend to be 'localized' early in the cell cycle and, as mentioned above, would display a tendency to differentiate into prestalk cells. It remains to be seen to what extent this might account for the ability of elevated Ca^{2+} to promote prestalk differentiation, and of lowered Ca^{2+} to promote prespore differentiation [9,24]. Interestingly, Ca^{2+} has been shown to regulate the transcription of certain genes, such as the rat prolactin gene [7]; growth in the presence of millimolar levels of another divalent cation (Mg^{2+}) represses the expression of Pho P-activated genes at the tran-

scriptional level and attenuates the virulence properties of wild-type *Salmonella* [25]. Likewise, Ca^{2+} may act as a signal that affects transcription of *clb1* in *Dictyostelium*. Also, as in other systems [13], there may be a reciprocal effect of cell cycle phase on calcium.

Acknowledgments

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References

[1] Bonner, J.T. (1967) The Cellular Slime Molds, 2nd edn. Princeton University Press, Princeton, NJ.
[2] Azhar, M., Kennady, P.K., Pande, G. and Nanjundiah, V. (1996) A Ca^{2+}-dependent early functional heterogeneity in amoebae of *Dictyostelium discoideum*, revealed by flow cytometry. Exp. Cell Res. 227, 344–351.
[3] Saran, S., Azhar, M., Manogaran, P.S., Pande, G. and Nanjundiah, V. (1994) The level of sequestered calcium in vegetative amoebae of *Dictyostelium discoideum* can predict post-aggregative cell fate. Differentiation 57, 163–169.
[4] Newell, P.C., Malchow, D. and Gross, J.D. (1995) The role of calcium in aggregation and development of *Dictyostelium*. Experientia 51, 1155–1165.
[5] Weijer, C.J., Duschl, G. and David, C.N. (1984) A revision of the *Dictyostelium discoideum* cell cycle. J. Cell Sci. 70, 111–131.
[6] Weeks, G. and Weijer, C.J. (1994) The *Dictyostelium* cell cycle and its relationship to differentiation. FEMS Microbiol. Lett. 124, 123–130.
[7] Preston, G.M., Billis, W.M. and White, B.A. (1990) Transcriptional and posttranscriptional regulation of the rat prolactin gene by calcium. Mol. Cell. Biol. 10, 442–448.
[8] Moreau, M., Leclerc, C., Gualandaris-Parisot, L. and Duprat, A.-M. (1994) Increased internal Ca^{2+} mediates neural induction in the amphibian embryo. Proc. Natl. Acad. Sci. USA 91, 12639–12643.
[9] Baskar, R. (1996) Early Commitment of Cell Types and a Morphogenetic Role for Calcium in *Dictyostelium discoideum*. Ph.D. Thesis, Indian Institute of Science, Bangalore.
[10] Nasmyth, K. (1996) Viewpoint: Putting the cell cycle in order. Science 274, 1643–1645.
[11] Luo, Q., Michaelis, C. and Weeks, G. (1994) Expression of truncated cyclin B gene arrests *Dictyostelium* cell division during mitosis. J. Cell Sci. 10, 3105–3114.
[12] Luo, Q., Michaelis, C. and Weeks, G. (1995) Cyclin B and Cdc2 expression and Cd2 kinase activity during *Dictyostelium discoideum* differentiation. DNA Cell Biol. 14, 901–908.
[13] Whitaker, M. and Patel, R. (1990) Calcium and cell cycle control. Development 108, 525–542.
[14] Pande, G., Kumar, A.N. and Manogaran, P.S. (1996) Flow cytometric study of changes in the intracellular free calcium during the cell cycle. Cytometry 24, 55–63.
[15] Watts, D.J. and Ashworth, J.M. (1970) Growth of myxamoebae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. Biochem. J. 119, 171–174.
[16] Krefft, M. and Weijer, C.J. (1989) Expression of a cell surface antigen in *Dictyostelium discoideum* in relation to the cell cycle. J. Cell Sci. 93, 199–204.
[17] Tirlapur, U.K., Gross, J. and Nanjundiah, V. (1991) Spatial variation of sequestered calcium in multicellular stage of *Dictyostelium discoideum* as assayed by chlortetracycline fluorescence. Differentiation 48, 137–146.
[18] Chomczynsky, P. and Sacchi, N. (1987) Single-step method of RNA isolation by a acidic guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[20] Michaelis, C. and Weeks, G. (1992) Isolation and characterization of a *cdc2* cDNA from *Dictyostelium discoideum*. Biochim. Biophys. Acta 1179, 117–124.
[21] Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6–13.
[22] Ceccarelli, A., McRobbie, S.J., Jermyn, K.A., Duffy, K., Early, A. and Williams, J.G. (1987) Structural and functional characterization of a *Dictyostelium* gene encoding a DIF-inducible, prestalk-enriched mRNA sequence. Nucleic Acids Res. 15, 7463–7476.
[23] Schlatterer, C. and Schaloske, R. (1996) Calmidazolium leads to an increase in the cytosolic Ca^{2+} concentration in *Dictyostelium discoideum* by induction of Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+}. Biochem. J. 313, 661–667.
[24] Kubohara, Y. and Okamoto, K. (1994) Cytoplasmic Ca^{2+} and H^{+} concentrations determine cell fate in *Dictyostelium discoideum*. FASEB J. 8, 869–874.
[25] Vescovi, E.G., Soncini, F.C. and Groisman, E.A. (1996) Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell 84, 165–174.