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Calmodulin is required for cell-cycle progression during G_1 and mitosis

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In order to examine the consequences of a transient increase or decrease in intracellular calmodulin (CaM) levels, two bovine-papilloma-virus (BPV)-based expression vectors capable of inducibly synthesizing CaM sense (BPV-MCM) or anti-sense (BPV-CaMAS) RNA have been constructed and used to stably transform mouse C127 cells. Upon addition of Zn^{2+}, cells containing the BPV-MCM vector have transiently increased CaM mRNA and protein levels. Cells carrying the BPV-CaMAS vector transiently produce CaM anti-sense RNA resulting in a significant decrease in intracellular CaM concentration. Increased CaM caused a transient acceleration of proliferation, while the anti-sense RNA induced decrease in CaM caused a transient cell cycle arrest. Flow cytometric analysis showed that progression through G_1 and mitosis was affected by changes in CaM levels. These data indicate that CaM levels may limit the rate of cell-cycle progression under normal conditions of growth.

Key words: calmodulin/cell cycle/G_1 phase/mitosis/BPV

Introduction

The mechanisms involved in regulating cell growth and cell-cycle progression are not well understood. Several studies have demonstrated roles for growth factors, hormones, cyclic nucleotides and ions in the control of cell proliferation. Calcium has long been recognized as an important component in regulating cell growth. Not only is Ca^{2+} absolutely required for cell viability, but progression through G_1 and mitosis are particularly sensitive to the intracellular Ca^{2+} concentration (Hazelton *et al.*, 1979; Izant, 1983).

Calmodulin (CaM), the major Ca^{2+} receptor in non-muscle and smooth muscle eukaryotic cells mediates many Ca^{2+}-dependent events (Means *et al.*, 1982). Genetic analysis in yeasts has shown that CaM is essential for cell viability (Davis *et al.*, 1986; Takeda and Yamamoto, 1987). In addition, CaM has been implicated as a regulator of cell proliferation. Pharmacological agents which selectively antagonize CaM function *in vitro* block cell-cycle progression both at the G_1/S boundary and during G_2/M (Chafouleas *et al.*, 1982; Sasaki and Hidaka, 1982; Eilam and Chernichovsky, 1988) and may cause cell cycle withdrawal and differentiation (Veigl *et al.*, 1986). Unfortunately, interpretation of these observations is hampered by the fact that *in vivo* these drugs also interact with targets other than CaM (e.g. protein kinase C) (Schatzman *et al.*, 1983). However, the intracellular CaM concentration doubles abruptly at the G_1/S boundary in Chinese hamster ovary (CHO) cells and mouse C127 cells (Chafouleas *et al.*, 1982; C.D.Rasmussen and A.R.Means, unpublished observations). This increase is temporally correlated with initiation of DNA synthesis consistent with a role for CaM at the G_1/S boundary (Chafouleas *et al.*, 1982, 1984). We have previously over-expressed a chicken CaM mini-gene in mouse C127 cells using a bovine-papilloma-virus (BPV)-based eukaryotic expression vector system. Constitutive elevation of intracellular CaM levels causes a shortening of the cell cycle due to reduction in the length of G_1, providing more direct evidence that the rate of cell proliferation may be sensitive to intracellular CaM concentration (Rasmussen and Means, 1987).

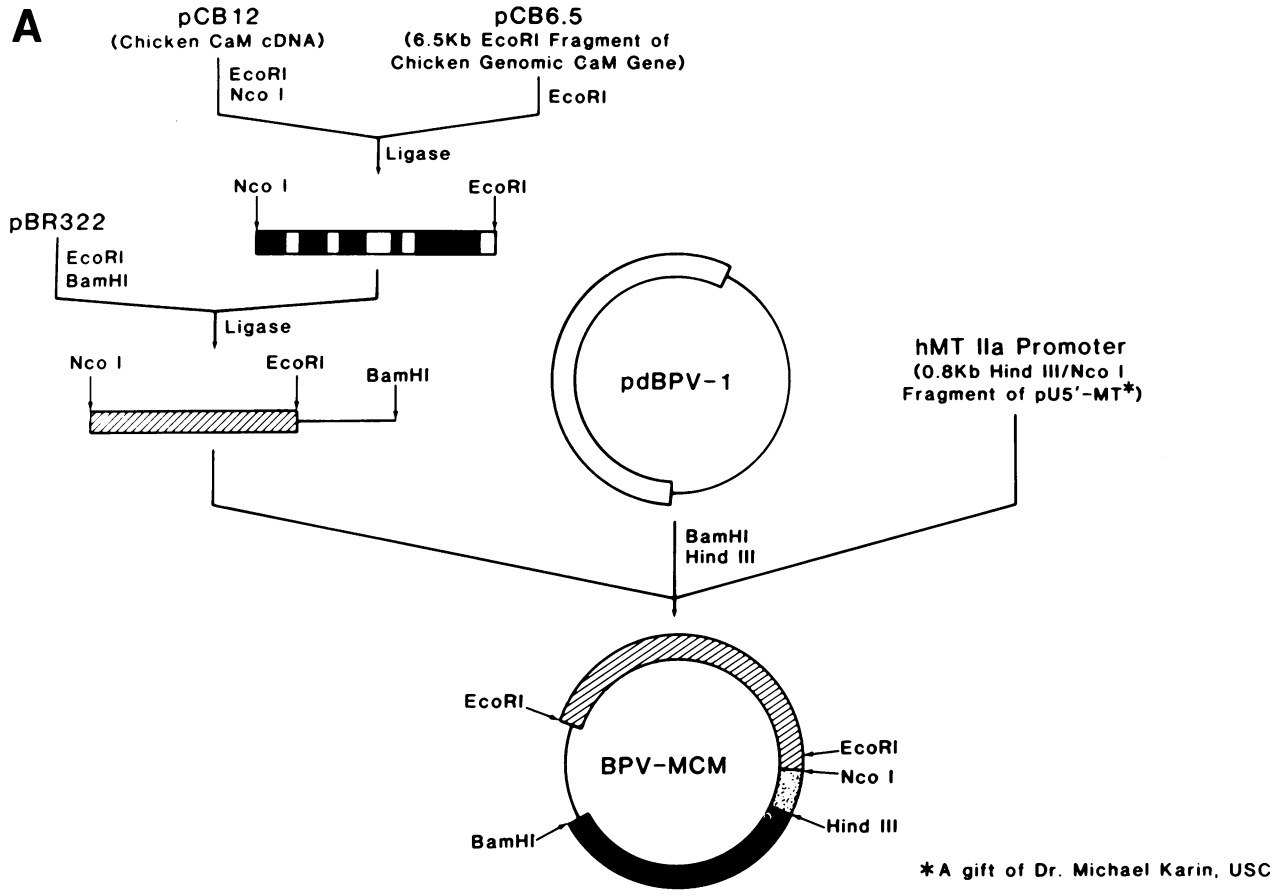
This study was designed to ask whether transient increases or decreases in intracellular CaM levels are sufficient to accelerate or inhibit cell-cycle progression. The experimental approach taken has been to utilize two BPV-based expression vectors which inducibly express either CaM sense or anti-sense RNA in stably transformed mouse C127 cell lines. In both the CaM sense and anti-sense vectors transcription is regulated by a Zn^{2+}-inducible metallothionein (MT) promoter. These promoters were chosen because high level transcription can be induced at Zn^{2+} concentrations which do not adversely affect the growth of C127 cells. In addition, the induction response is transient so that the induced RNA returns to control levels even in the continued presence of Zn^{2+} (Durnam and Palmiter, 1981; Yagle and Palmiter, 1985). This allows a direct correlation to be made between Zn^{2+}-induced changes in CaM levels and a cellular response.

The results show that cell cycle progression is affected by either a transient increase or decrease in CaM levels. Detailed analysis reveals that progression through both G_1 and mitosis are sensitive to changes in intracellular CaM concentration. These data suggest that under normal growth conditions CaM concentration may be rate determining for cell-cycle progression.

Results

Inducible expression of a calmodulin mini-gene

We first wished to determine whether an inducible, transient elevation of CaM levels would cause effects on cell-cycle progression similar to those previously described in response to constitutive increases (Rasmussen and Means, 1987). To accomplish this a BPV-based eukaryotic expression vector, in which transcription of the chicken CaM gene could be transiently increased, was constructed and used to stably transform mouse C127 cells. The vector, BPV-MCM (Figure 1A) consists of (i) a 6.6-kb chicken CaM mini-gene (Rasmussen and Means, 1987), (ii) a 0.85-kb fragment of the human MT-IIa gene promoter, containing the response elements for induction by heavy metals and glucocorticoids (obtained from M.Karin, USC) (Karin *et al.*, 1984), and (iii)


A
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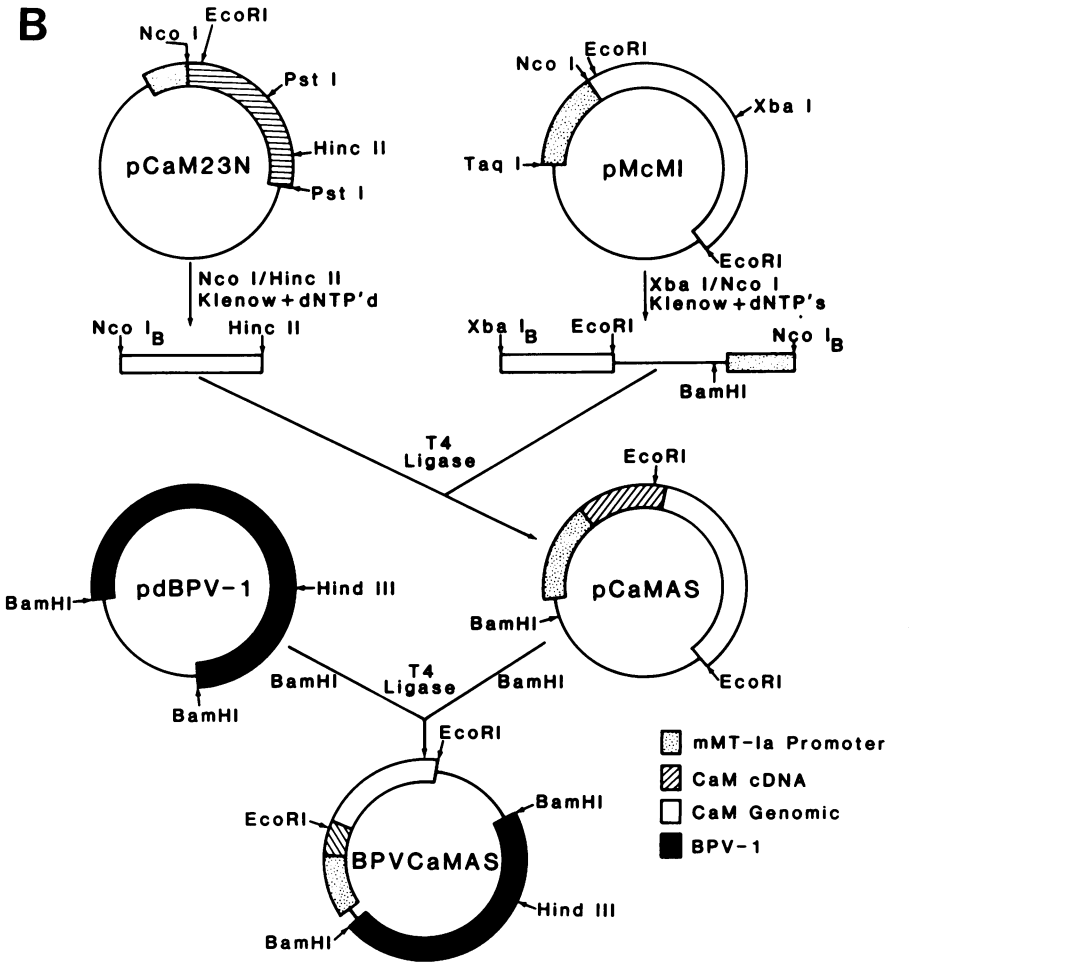
(Chicken CaM cDNA)
EcoRI
Nco I
pCB6.5
(6.5Kb EcoRI Fragment of
Chicken Genomic CaM Gene)
EcoRI
Ligase
Nco I
EcoRI
pBR322
EcoRI
BamHI
Ligase
Nco I
EcoRI
BamHI
pdBPV-1
hMT IIa Promoter
(0.8Kb Hind III/Nco I
Fragment of pU5'-MT*)
BamHI
Hind III
EcoRI
BPV-MCM
EcoRI
Nco I
Hind III
BamHI
*A gift of Dr. Michael Karin, USC


B
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EcoRI
Pst I
pCaM23N
Hinc II
Pst I
Nco I/Hinc II
Klenow+dNTP'd
Nco I B
Hinc II
Nco I
EcoRI
Xba I
Taq I
pMcMI
EcoRI
Xba I/Nco I
Klenow+dNTP's
Xba I B
EcoRI
Nco I B
BamHI
T4
Ligase
EcoRI
pdBPV-1
BamHI
Hind III
BamHI
pCaMAS
BamHI
EcoRI
BamHI
T4
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BamHI
EcoRI
EcoRI
BamHI
BPVCaMAS
Hind III
BamHI
mMT-Ia Promoter
CaM cDNA
CaM Genomic
BPV-1

the 69% transforming region of BPV-1 in the pBR322 derivative pML2, to allow propagation in *Escherichia coli* (Sarver *et al.*, 1981). The hMT-IIa promoter was selected as it contains a unique *Nco*I restriction site at the initiation ATG, as does the chicken CaM mini-gene used in these studies. The ligation of the CaM mini-gene to the MT-IIa DNA regenerates a consensus translation initiation site (CCATGG) and maintains the same spatial orientation between MT-IIa promoter elements and the translation start site.

Low passage number C127 cells were transfected with BPV-MCM DNA using the calcium-phosphate-transfection procedure as previously described (Rasmussen *et al.*, 1987). Transformed foci were selected 10–14 days after transfection and propagated as individual clonal cell lines (designated MCM cells). Those cell lines in which the BPV-MCM plasmid was maintained as a nuclear episome were assayed for Zn^{2+}-inducible expression of the vector-borne chicken CaM gene, using a specific hybridization probe obtained from the unique 3′ non-translated region (Putkey *et al.*, 1985). Of the 20 cell lines initially isolated, four maintained the BPV-MCM DNA as an episome and produced increased levels of chicken CaM mRNA in response to Zn^{2+}. Of these four lines, one (MCM-4) consistently produced a 7- to 8-fold increase in vector-derived CaM mRNA and was selected for further characterization. The BPV-MCM vector was present at ~40 copies/cell as judged from Southern blot analysis of DNA from MCM-4 cells (data not shown).

The kinetics of vector-derived CaM mRNA accumulation in MCM-4 cells in response to Zn^{2+} were examined. $ZnSO_4$ was added to exponentially growing MCM-4 cells at a final concentration of 80 μM. This is the highest concentration of Zn^{2+} that could be added to a BPV-transformed control cell line (BPV-1) (Rasmussen and Means, 1987) without causing adverse effects on cell growth. Prior to adding Zn^{2+}, and at intervals following Zn^{2+} addition, cytoplasmic RNA was isolated from MCM-4 cells and CaM mRNA analysed by Northern blot and slot–blot hybridization (Figure 2). CaM mRNA levels were quantitated by scanning densitometry of the autoradiograms. Hybridization of the chicken-mRNA-specific probe to total cytoplasmic RNA from MCM-4 cells demonstrated that the appropriate 1.6-kb mRNA appeared in response to Zn^{2+}-induction (Figure 2A). The level of this mRNA increased nearly 8-fold within 6 h of Zn^{2+} addition (Figure 2B), followed by a decline to approximately twice the uninduced level, by 12 h. The transient response of the MT promoter used in the BPV-MCM vector to Zn^{2+} is consistent with previous observations of Yagle and Palmiter (1985). Hybridization of a CaM-coding sequence probe that recognizes both mouse and chicken CaM mRNAs showed that vector-derived CaM mRNA was sufficient to raise transiently the total cellular CaM mRNA levels by nearly 4-fold (Figure 2C).

Intracellular CaM levels were quantified by radioimmunoassay (RIA) (Chafouleas *et al.*, 1979), to determine if an increase in CaM accompanied the elevation in CaM mRNA in MCM-4 cells. Calmodulin levels in Zn^{2+}-treated BPV-1 cells were also measured to control for non-specific effects of Zn^{2+} on intracellular CaM concentration. Figure 3A shows that CaM levels rise in response to increased CaM mRNA levels, from an uninduced level of 400 ng/10^6 cells to 614 ng/10^6 cells. This increase (54% within 4 h) is significant since CaM levels only vary over a 2-fold range during the cell cycle (Chafouleas *et al.*, 1982; C.D.Rasmussen and A.R.Means, unpublished observations). The observation that CaM levels did not increase to the same extent as the vector-derived mRNA was not unexpected since we have previously observed the same result in cells which constitutively express the CaM mini-gene used in this study (Rasmussen and Means, 1987).

Increased calmodulin levels accelerate cell cycle progression

We next determined whether cell growth and cell-cycle progression are affected by transient changes in intracellular CaM concentration. Exponentially growing cultures of MCM-4 and BPV-1 cells were replicate plated at low density. Twenty-four hours later, Zn^{2+} was added to half the cultures and cell growth of the treated and untreated cells monitored. Addition of Zn^{2+} produced no significant change in the growth of control BPV-1 cells (Figure 4A). In contrast, a transient increase in cell growth rate was observed in Zn^{2+}-treated MCM-4 cells (Figure 4B). The rate of proliferation of MCM-4 cells was accelerated within 3 h following the addition of Zn^{2+}, and returned to the control level after 8 h. This change in growth rate was temporally correlated with the changes in CaM levels observed in MCM-4 cells in response to Zn^{2+}. These data show that a transient increase in CaM levels is sufficient to increase the rate at which cells traverse the cell cycle.

Flow microfluorometric (FMF) analysis was used to determine at which point in the cell cycle increased CaM levels exerted an effect. Cells were stained with the fluorochrome propidium iodide, the DNA content of individual cells determined fluorometrically, and a profile representing the frequency distribution of cellular DNA content of exponentially growing cells was generated. To analyse changes in the relative distribution of cells within the cell cycle a software program for subtraction analysis (part of the MDADS system; Coulter Electronics) was utilized. In this procedure, the FMF profile obtained for untreated, exponentially growing BPV-1 cells was subtracted from the profile obtained both for untreated MCM-4 cells and at various times (4, 8 and 12 h) following the addition of Zn^{2+}. The resulting difference profile provides a quantitative index of both the magnitude and distribution within the cell cycle of differences between two cell populations. For example, if there are more MCM-4 cells at a particular cell cycle stage as compared with the BPV-1 population a peak will be observed. The relative DNA

Fig.1. Construction of BPV expression vectors. (**A**) BPV-MCM was constructed as follows. The constructon of the CaM mini-gene, which is contained in a 9.6-kb *Nco*I–*Bam*HI fragment has been described previously (Rasmussen and Means, 1987). This fragment was ligated, in a single step, to a 0.8-kb *Nco*I–*Hin*dIII fragment of the hMT-IIa promoter and a 5.5-kb *Bam*Hi–*Hin*dIII fragment of the BPV-I genome, to produce BPV-MCM. (**B**) BPV-CaMAS was constructed as follows. A 1.0-kb *Nco*I–*Hin*dIII fragment of the chicken CaM cDNA, contained in the vector pCaM23N (Putkey *et al.*, 1987) was blunt-ended and ligated to a blunt-ended *Nco*I–*Xba*I fragment of the plasmid pMcMI, which contains the mMT-I promoter and 3.0 kb of the 3′ untranslated region of the chicken CaM gene, and a clone which contained the chicken CaM cDNA in reverse orientation selected. This intermediate vector, pCaMAS, was then digested with *Bam*HI and ligated to a *Bam*HI fragment containing the entire BPV-1 genome to produce BPV-CaMAS. A plasmid which contained the BPV-1 genome in the orientation shown was used for transfection.

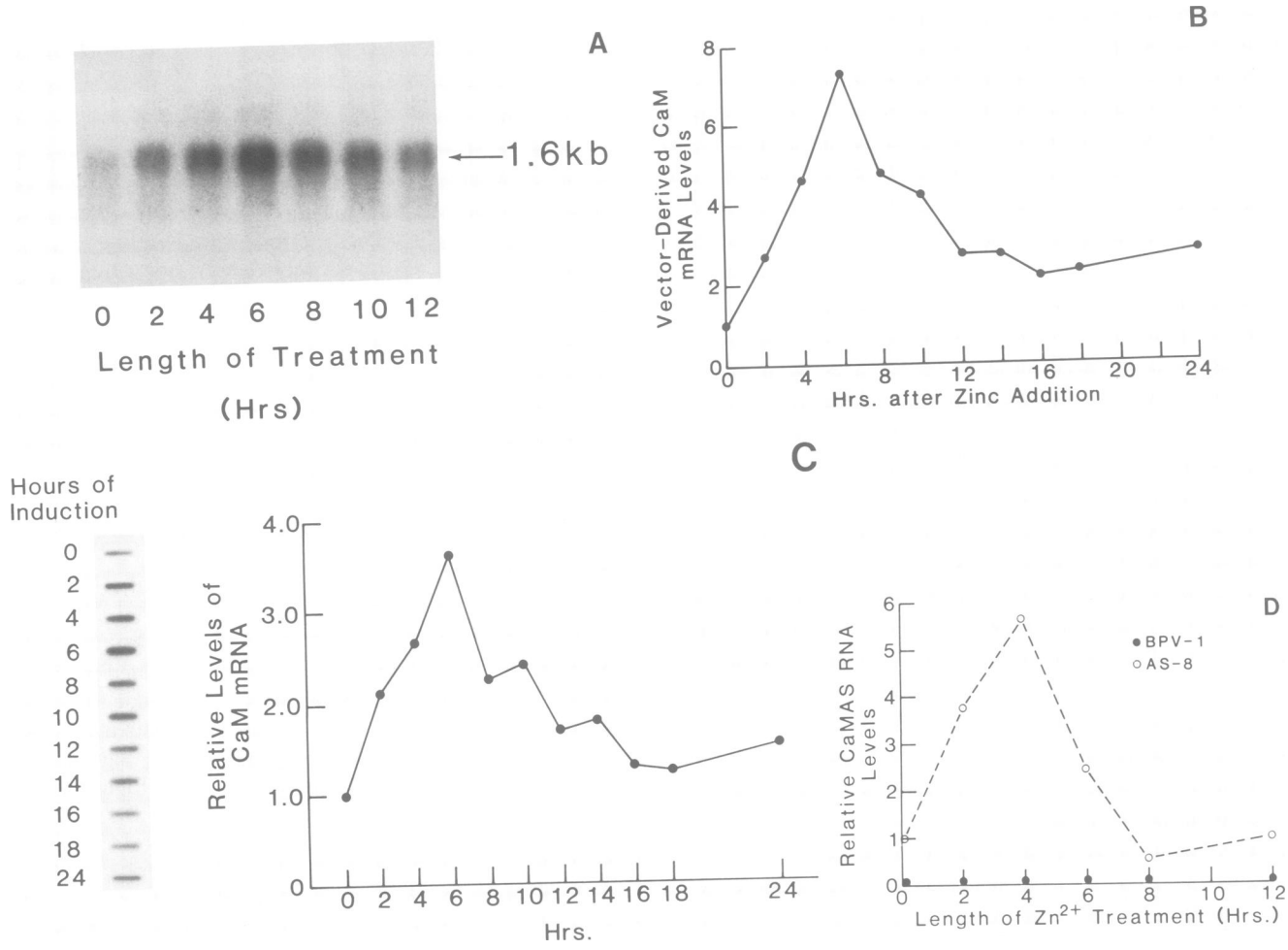

Fig. 2. CaM sense and anti-sense RNA in transformed C127 cells. (**A**) Northern analysis of MCM-4 cells during Zn^{2+} induction. Equal amounts (10 μg) of cytoplasmic RNA isolated from MCM-4 cells treated with 80 μM $ZnSO_4$ for various times was electrophoresed on 1.2% agarose–formaldehyde gels, and transferred to a BioTrans filter (ICN) as described in the Materials and methods. The blot was probed with a cDNA fragment derived from the 3′ untranslated region of the chicken CaM cDNA (Putkey *et al.*, 1985). (**B**) Quantitation of vector-derived CaM mRNA in MCM-4 cells. The levels of vector-derived CaM mRNA were quantitated by scanning densitometry of the blot shown in (A). (**C**) Quantitation of total CaM mRNA in MCM-4 cells. The levels of total CaM mRNA during Zn^{2+} induction of MCM-4 cells was determined by slot–blot hybridization. Equal amounts (5 μg) of cytoplasmic RNA from the same experiment used for (A) were immobilized on a Biotrans filter using a Minifold II slot–blot apparatus (Schleicher and Schuell) and probed with a chicken CaM cDNA fragment derived from the coding region, which recognizes both chicken and endogenous mouse CaM mRNA. The inset shows the autoradiogram of the dot blot, while the graph represents the quantitation of the autoradiographic signal by scanning densitometry. (**D**) Analysis of CaM anti-sense RNA in AS-8 cells. Equal amounts of cytoplasmic RNA from Zn^{2+}-treated AS-8 and BPV-1 cells was isolated and transferred to a Biotrans filter using a dot–blot apparatus. The filter was probed with a sense strand RNA synthesized as described in Materials and methods. After hybridization and washing the amount of probe hybridized was determined by scintillation spectrometry of the excised dots. The background hybridization to the filter alone was determined by scintillation spectrometry of an equal sized portion of the filter which did not contain RNA.

content of the cells in the peak indicates in which cell-cycle phase the difference occurred. A value of zero (i.e. baseline) indicates that either there was no difference or there were relatively more BPV-1 cells than MCM-4 cells with that DNA content. As a control for non-specific effects of Zn^{2+} on distribution of cells in the cell cycle, the same analysis was applied to Zn^{2+}-treated BPV-1 cells.

The FMF profiles of untreated exponentially growing BPV-1 and MCM-4 cells are shown in Figure 5A and E. The difference profile of untreated MCM-4 cells indicates there are more cells with DNA contents characteristic of both G_1 and G_2/M cells as compared with BPV-1 cells (Figure 5F), amounting to 7.8% of the population. This was expected as MCM-4 cells have a longer G_1 period and an increased mitotic index relative to BPV-1 cells (C.D.Rasmussen and A.R.Means, unpublished observations).

Addition of Zn^{2+} produced a considerable change in the relative distribution of MCM-4 cells within the cell cycle. Four hours after Zn^{2+} addition there was a significant increase in the relative number of MCM-4 cells with DNA contents characteristic of cells in early S-phase (Figure 5G). By 8 h this population of cells progressed through S resulting in a relative increase in the number of cells with DNA content characteristic of mid-to-late-S-phase cells (Figure 5H), and by 12 h after Zn^{2+} addition these cells had completed mitosis resulting in an increase in the relative number of MCM-4 cells in G_1. In contrast, Zn^{2+} treatment of BPV-1 cells caused no significant redistribution of cells within the cell cycle (Figure 5B–D). While the differences observed for BPV-1 cells were never >5% of the total cell population, up to 18.5% of the MCM-4 cell population was affected, accounting for the increase in cell number (~20%) observed in Zn^{2+}-treated MCM-4 cells. Together these data demonstrate that CaM alters the rate of cell-cycle progression at two points in the cell cycle, G_1 and G_2/M. The FMF experiments indicate that transient elevation of CaM levels

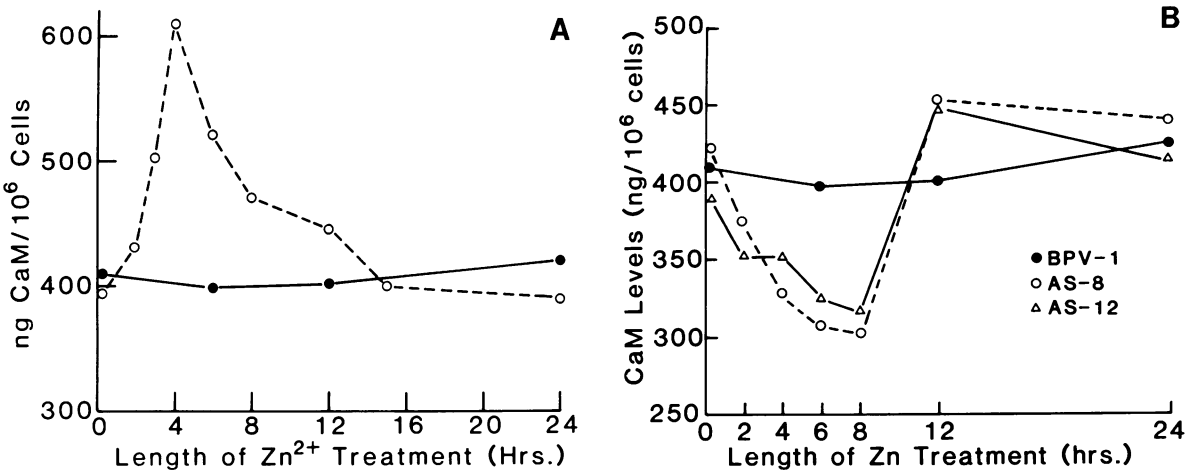

Fig. 3. CaM levels in transformed C127 cells. (**A**) CaM levels during Zn^{2+} treatment of MCM-4 cells. CaM levels were determined in Zn^{2+}-treated MCM-4 and BPV-1 cells by RIA as described (Chafouleas *et al.*, 1979). (**B**) CaM levels during Zn^{2+} treatment of AS-8 and AS-12 cells. CaM levels were determined in Zn^{2+}-treated AS-8 and AS-12 cells as described above. The data for BPV-1 cells shown in (A) is included for comparison.

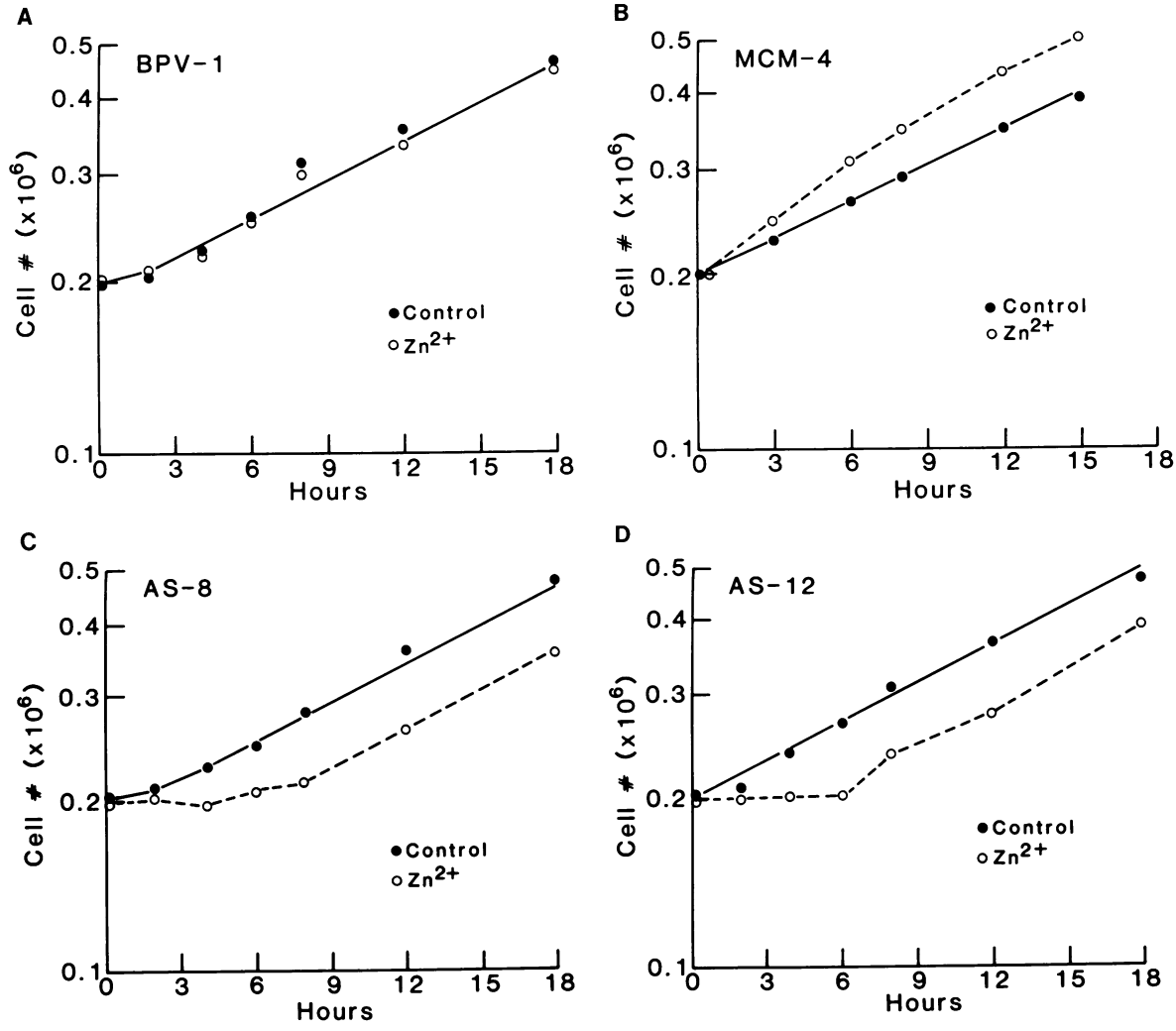

Fig. 4. Effect of Zn^{2+} on growth of transformed C127 cells. The growth of cells with or without added Zn^{2+} was determined as described in the text. Cell number determinations in replicate samples showed that overall variation was consistently <5%. (**A**) BPV-1 cells; (**B**) MCM-4 cells; (**C**) AS-8 cells; (**D**) AS-12 cells. Filled circles are untreated cells, open circles are cells treated with 80 μM $ZnSO_4$.

resulted in an increased rate of entry of cells into S phase. The observation that cell-division rate increases soon after addition of Zn^{2+} suggests that progression through G_2/M is also transiently accelerated by increased CaM levels.

Inducible expression of a CaM anti-sense RNA

Earlier studies examining the effect of anti-CaM drugs on cell cycle progression suggested that a reduction of CaM levels would inhibit cell proliferation. To examine the

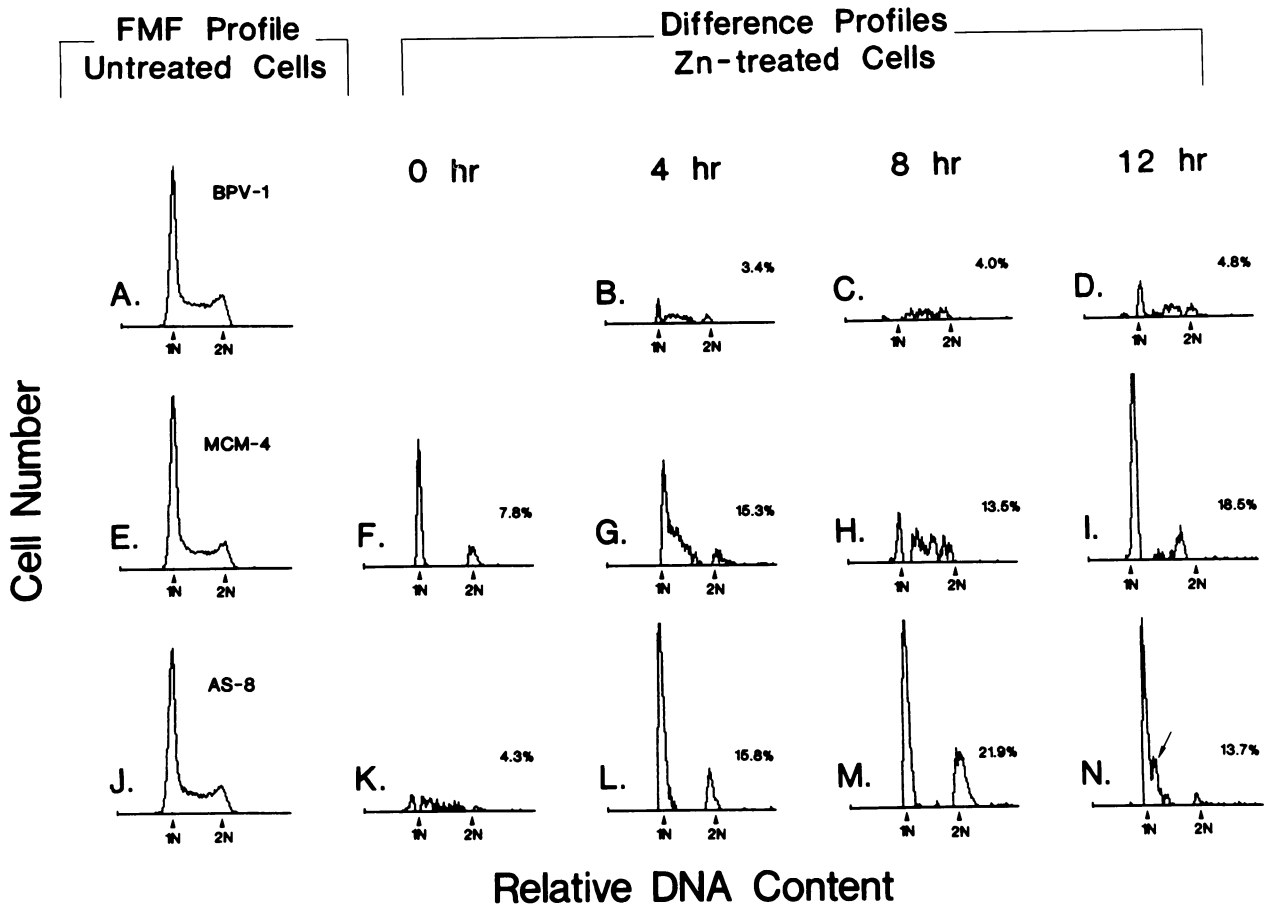

Fig. 5. Flow cytometric analysis of Zn^{2+}-treated cells. Cells were analysed by FMF as described in the text and Materials and methods. (**A**–**D**) BPV-1 cells; (**E**–**I**) MCM-4 cells; (**I**–**N**) AS-8 cells. (**A, E** and **J**) FMF profiles of exponentially growing cells. Difference profiles of cells untreated (**F** and **K**) and cells treated for 4 h (**B, G** and **L**), 8 h (**C, H** and **M**) and 12 h (**D, I** and **N**) with 80 μM $ZnSO_4$.

consequence of a specific reduction of CaM levels on cell proliferation, a BPV-based vector capable of transiently synthesizing CaM anti-sense RNA (CaMAS RNA) was constructed. The vector BPV-CaMAS (Figure 1B) consists of a 1.0-kb *Nco*I–*Hin*cII fragment of a chicken CaM cDNA (Putkey *et al.*, 1985) blunt-ended with Klenow enzyme and ligated, in reversed orientation, 3′ to the Zn^{2+}-inducible mouse MT-I (mMT-I) promoter (obtained from D.Hamer, NIH) (Durnam *et al.*, 1980). Further 3′ to the reversed CaM cDNA sequences a 3.0-kb segment of the 3′ untranslated region of the chicken CaM gene (from the vector BPV-CM) (Rasmussen and Means, 1987) was placed to provide signals for transcription termination and polyadenylation. BPV-CaMAS also contains the BPV-1 genome in an orientation which places the BPV enhancer sequences as far as possible from the mMT-I promoter to ensure that anti-sense RNA transcription would be as low as possible in the absence of Zn^{2+}. C127 cells were transfected with BPV-CaMAS DNA and transformed foci selected 14 days after transfection. Of the 12 foci selected and cloned as cell lines (designated AS cells), two maintained the plasmid at high copy number (~50–100 copies/cell) as an un-rearranged episome, and were retained for detailed analysis (designated AS-8 and AS-12).

The kinetics of cytoplasmic CaMAS RNA accumulation in response to 80 μM $ZnSO_4$ were examined in AS-8 cells by RNA dot–blot hybridization. The hybridization probe was a ^{32}P-labelled sense strand RNA produced by T7 RNA polymerase transcription of an *Eco*RI–*Pst*I fragment of the chicken CaM cDNA present in the BPV-CaMAS vector, subcloned into the riboprobe vector pGEM-1 (Promega Biotech). As a control for non-specific hybridization of the riboprobe to cellular RNA, equal amounts of cytoplasmic RNA from identically treated BPV-1 cells were included on the dot blot. After hybridization, the dots were cut from the filter and the amount of hybridized probe quantitated by scintillation spectrometry. As shown in Figure 2c, the sense strand RNA failed to hybridize to RNA from control BPV-1 cells. CaMAS RNA was detectable in cytoplasmic RNA from AS-8 cells, and increased 6-fold within 4 h in response to Zn^{2+}. As was observed for Zn^{2+}-induced synthesis of CaM sense RNA, anti-sense RNA levels were transiently elevated, returning to control levels by 8 h. The kinetics of anti-sense RNA accumulation in response to Zn^{2+} are consistent with earlier studies which examined the response of the mMT-I promoter to heavy metal induction (Durnam and Palmiter, 1981).

CaM levels were measured during the course of Zn^{2+}-induction to determine if the presence of AS RNA decreased intracellular CaM levels. The results show that CaM levels declined in both AS-8 and AS-12 cells in response to Zn^{2+}, reaching a minimum of nearly 300 ng/10^6 cells by 8 h (Figure 3b). This represents a 30% decline in CaM, to levels similar to that observed in G_1 cells (250 ng/10^6 cells). The half-life of this decline was ~14 h, similar to that observed in virally transformed 3T3

cells (Chafouleas *et al.*, 1981). CaM levels recover to pre-induction levels by 12 h, concomitant with the disappearance of CaMAS RNA. Since expression of anti-sense RNA resulted in a significant depression of CaM levels, cell proliferation in Zn^{2+}-treated AS cells was examined.

Reduced CaM levels cause transient cell cycle arrest

As shown in Figure 4A, the addition of $ZnSO_4$ to BPV-transformed controls had no significant affect on cell growth. However, in both AS-8 and AS-12 cells, Zn^{2+} treatment caused a transient cessation of cell proliferation (Figure 4C and D). This effect was first observed between 2 and 4 h after Zn^{2+} addition and cell proliferation did not resume until 8−12 h. Both the block of cell proliferation and subsequent recovery were temporally correlated with the observed changes in CaM levels during the course of Zn^{2+} induction of CaM anti-sense RNA.

The observation that the rapid reduction in CaM levels was correlated with a proliferation block suggested that progression through the later stages of the cell cycle (G_2/M) was being inhibited. Therefore, FMF analysis was used to determine where cell-cycle arrest had occurred. The FMF profile of untreated exponentially growing AS-8 cells (Figure 5J) is nearly identical to that of BPV-1 cells (Figure 5A). The difference profile of untreated cells revealed no significant difference in the distribution of AS-8 relative to BPV-1 cells within the cell cycle (Figure 5K). While Zn^{2+} treatment was previously shown to have no significant effect on the distribution of BPV-1 cells within the cell cycle, analysis of Zn^{2+}-treated AS-8 cells showed that within 4 h a significant portion of the population (15.8%) had accumulated in both the G_1 and G_2/M compartments of the cell cycle. This effect was maximal (involving 21.9% of the population) 8 h after addition of Zn^{2+} (Figure 5M). By 12 h the 2*N* (G_2/M) cohort of cells disappeared as they recovered from cell-cycle arrest and completed mitosis. In addition, a distinct group of cells with a DNA content characteristic of early S phase cells was observed (arrow; Figure 5N). This result is consistent with the recovery of a cell population which had been arrested at the G_1/S boundary. Thus Zn^{2+} treatment of AS cells resulted in a transient blockage of cell-cycle progression at two points in the cell cycle, G_1 and in G_2/M.

Growth-arrested AS cells are blocked at metaphase

Since cells in G_2 and mitosis have identical DNA content (2*N*), the location of the second blockpoint cannot be resolved by flow cytometry. To differentiate between these two cell-cycle phases, the mitotic index of BPV-1 and AS-8 cells was determined during the course of Zn^{2+} treatment. As shown in Figure 6, Zn^{2+} treatment caused no change in the mitotic index of BPV-1 cells. However, Zn^{2+} treatment of AS-8 cells resulted in an increase in the proportion of cells in mitosis, from 6.9 to 16.8% by 8 h, indicating that growth arrest results from an arrest in mitosis. The mitotic index of AS-8 cells subsequently declined by 12 h, concomitant with the resumption of cell proliferation and release of cells from the mitotic block.

In order to determine the point in mitosis where blockage occurred, the relative distribution of cells within each phase of mitosis was examined. Zn^{2+} treatment did not significantly change the distribution of BPV-1 cells within mitosis (Figure 7A). However, in AS-8 cells, the proportion

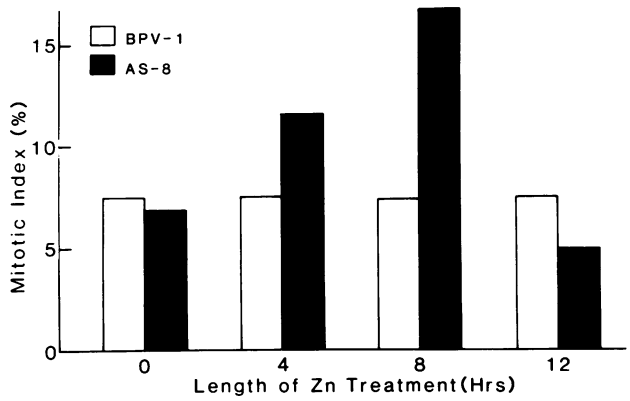

Fig. 6. Effect of Zn^{2+} treatment on the mitotic index. The mitotic index is the percentage of the cell population in mitosis and was determined by microscopic examination as described in Materials and methods. For each point at least 1000 cells were analysed. Open bar, BPV-1; filled bar, AS-8.

of cells in metaphase increased to nearly 60% of the mitotic population, while there was an almost complete absence of cells in anaphase (Figure 7B). These data suggest that progression of cells past metaphase of mitosis may be dependent on a critical CaM concentration.

Discussion

CaM has been suggested to regulate numerous intracellular processes (reviewed in Means *et al.*, 1982). Previous studies have provided evidence that CaM plays a role in the regulation of cell proliferation. The approach taken in this study has been to assess the effect of specifically changing intracellular CaM levels in order to define a role for CaM in the control of cell proliferation.

The observation that increased CaM in MCM cells accelerated cells past the G_1/S boundary, while a specific decrease in CaM levels in AS cells prevented progression into S phase indicates that one CaM-dependent control point exists in G_1. The data suggest that CaM regulates the rate of cell proliferation by controlling the rate at which cells enter S phase. The implication of these results is, that under normal growth conditions, the rate of proliferation will be regulated by intracellular CaM concentration. A number of previous studies on Ca^{2+} and CaM can be interpreted to support this hypothesis. It has long been recognized that Ca^{2+} is an important component in the regulation of cell proliferation (Whitfield *et al.*, 1976), and is required during the latter part of G_1 in both cycling and mitogenically stimulated cells (Hazelton *et al.*, 1979). The results are consistent with our contention that CaM mediates the Ca^{2+} control of entry into S phase. Disruption of the CaM gene in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has shown that CaM is essential for cell viability (Davis *et al.*, 1986; Takeda and Yamamoto, 1987). In *S.pombe*, germinated spores carrying a disrupted CaM are unable to complete a cell cycle suggesting that the residual amount of CaM per cell in G_1 is below the level required for proliferation. In CHO cells (Chafouleas *et al.*, 1982) and in BPV-transformed C127 cells (C.D.Rasmussen and A.R.Means, unpublished observations) CaM levels double abruptly at the G_1/S boundary, promoting the suggestion that an increase in CaM is required for cells to initiate DNA

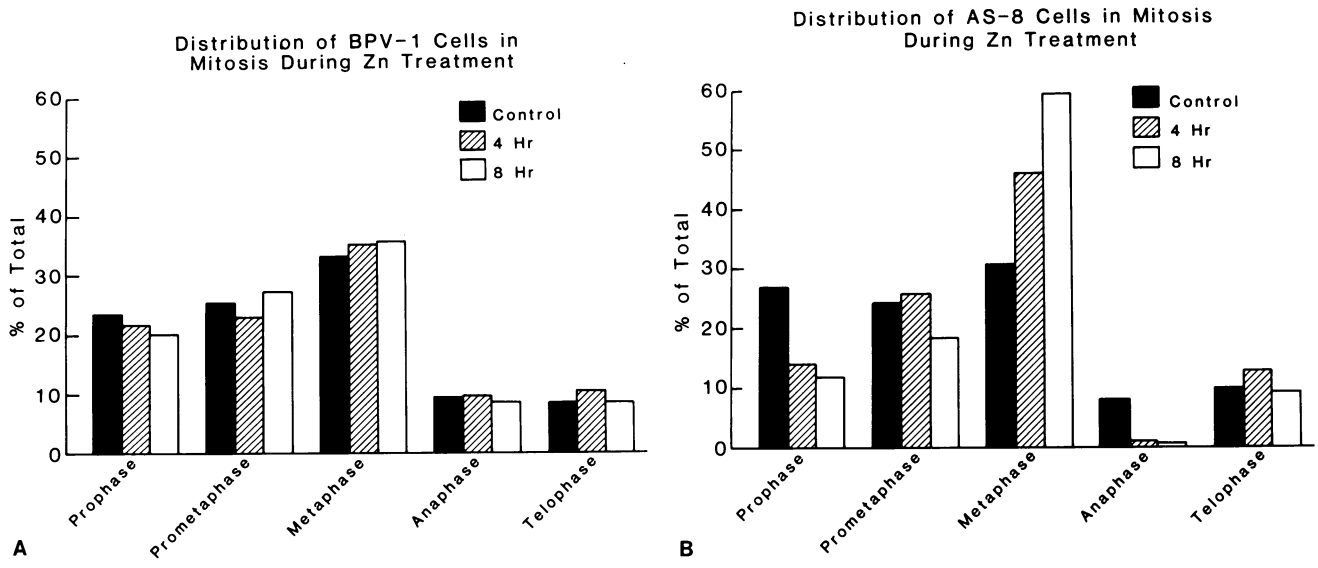

Fig. 7. Effect of Zn^{2+} treatment on the distribution of cells in mitosis. The distribution of cells within each compartment of mitosis was determined by microscopic examination. For each point, at least 150 mitotic cells were analysed. (**A**) BPV-1 cells; (**B**) AS-8 cells; filled bar, untreated cells; hatched bar, cells treated for 4 h; open bar, cells treated for 8 h.

synthesis and progress through S phase. Additional support for this supposition comes from pharmacological experiments which show that both mammalian and yeast cells are specifically and reversibly blocked in G_1 by treatment with CaM antagonist drugs at concentrations which do not affect cell viability (Chafouleas *et al.*, 1982; Sasaki and Hidaka, 1982; Eilam and Chernichovsky, 1988). In addition our recent results reveal that elevation of CaM levels by constitutive over-expression of a chicken CaM gene in mouse C127 cells results in a shortened cell cycle due to a reduction in the length of G_1 (Rasmussen and Means, 1987). The major challenge of future study will be to identify the molecular nature of the CaM-dependent control point present in G_1.

The observation that reduced CaM levels in AS cells results in metaphase arrest suggests that CaM is also required during mitosis for the metaphase–anaphase transition that results in movement of chromosomes to the spindle poles. Several studies are supportive of a role for both Ca^{2+} and CaM in this phase of mitosis. First both Ca^{2+} and CaM are concentrated in the centrosomal region of the mitotic spindle (Welsh *et al.*, 1978, 1979; Wolniak *et al.*, 1980). It is generally agreed that poleward movement of the chromosomes at anaphase requires the depolymerization of the spindle microtubules (Inoue and Ritter, 1975). Both the assembly and disassembly of microtubules are sensitive to Ca^{2+} (Weisenberg, 1972). Injection of Ca^{2+} into metaphase PtKl cells accelerates the onset of anaphase, whereas injection of EGTA results in delayed mitotic progression (Izant, 1983). Measurements of Ca^{2+} concentrations in the mitotic spindle *in vitro* using the fluorescent indicator fura-2 reveals that a transient 4- to 6-fold increase occurs coincident with the onset of anaphase (Poenie *et al.*, 1985, 1986; Ratan *et al.*, 1986). CaM has been shown to reduce the Ca^{2+} concentration required for depolymerization of microtubules *in vitro* and *in vivo* (Marcum *et al.*, 1978; Keith *et al.*, 1983) while the CaM inhibitor chlorpromazine has been demonstrated to block mitosis at metaphase (Boder *et al.*, 1983). Together these data suggest that a threshold level of CaM must be attained in order for cells to progress through G_1 and mitosis. It may be possible to analyse this in greater detail by altering the time of Zn^{2+} addition to mitotically synchronized cells during the cell cycle. Using this approach it should be possible to quantitatively determine the threshold level of CaM required for progression past the G_1/S boundary and through mitosis.

A putative role for CaM in mitosis may be to regulate microtubule stability. This could occur either by reducing the amount of Ca^{2+} required for microtubule depolymerization (Marcum *et al.*, 1978; Keith *et al.*, 1983), or through changes in Ca^{2+}/CaM-dependent phosphorylation of proteins associated with the mitotic apparatus. Tubulin, microtubule-associated proteins (MAPS) (Job *et al.*, 1983; Schulman, 1984; Sayhoun *et al.*, 1986; Wandosell *et al.*, 1986) and a recently described 62-kd protein present in the mitotic apparatus have been reported to be substrates for Ca^{2+}-CaM-dependent protein kinases. In fact, *in vivo* phosphorylation of the 62-kd protein occurs only during mitosis, and appears to be correlated with the destabilization of microtubules (Dinsmore and Sloboda, 1988). It is our view that regulation of a protein phosphorylation cascade is the most likely role for CaM in mitosis.

Cyclic changes in the phosphorylation of specific proteins (mostly of unknown function) is an established feature of the cell cycle (Westwood *et al.*,1985; Morla and Wang, 1986; Tripp *et al.*, 1986; Dessev *et al.*, 1988; Lohka *et al.*, 1988; Pognonec *et al.*, 1988). In yeast, control of cell-cycle progression through both G_1 and mitosis is regulated by genes (the *cdc28* gene of *S.cerevisiae* and the *cdc2* gene of *S.pombe*) which have been shown to be protein kinases (Reed *et al.*, 1985; Simanis and Nurse, 1986; Russell and Nurse, 1987). A protein immunologically similar to *cdc2* (termed p34) is present in extracts from HeLa cells. It has recently been shown that *cdc2*/p34 is a component of the maturation-promoting-factor (MPF) activity from *Xenopus* (Dunphy *et al.*, 1988; Gautier *et al.*, 1988). MPF has previously been shown to actively induce mitosis in eggs and embryos (Newport and Kirschner, 1984) and to contain protein-kinase activity (Lohka *et al.*, 1988). In HeLa cells, *cdc2*/p34 is part of a complex that contains two additional proteins, p13 (the

homologue of the *S.pombe suc1*$^+$ gene) and a 62-kd protein which is phosphorylated specifically in mitotic cells (Draetta *et al.*, 1987; Draetta and Beach, 1988). The possibility exists that the 62-kd phosphoprotein present in the *cdc2*/p34 complex may be homologous to the 62-kd mitotic-apparatus-associated protein which has been shown to be phosphorylated by a Ca^{2+}-CaM dependent protein kinase (Dinsmore and Sloboda, 1988), suggesting a connection between CaM and the *cdc2*/MPF complex. The availability of cell lines in which CaM levels can be manipulated allows us to test whether changes in CaM levels can alter either the activity of *cdc2*/p34, or its association with other proteins.

Materials and methods

Culturing mouse C127 cells

Mouse C127 cells are a non-transformed cell line derived from a mouse mammary tumour (Lowy *et al.*, 1978) and were obtained from Dr Dean Hamer at NIH. The cells are routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) (Gibco) at 37°C in a humidified, 95% air/5% CO_2 atmosphere and passaged when 70–80% confluent. Cells were maintained in T-flasks (75 cm^2) (Falcon Plastics) while cells used in experiments were grown in plastic Petri dishes (either 60 or 100 mm) (Falcon Plastics).

Transfection of cells and selection of transformed foci

Early passage C127 cells were transfected with either BPV-MCM or BPV-CaMAS DNA (intact plasmid form) as previously described (Rasmussen *et al.*, 1987). Transformed cells were selected on the basis of focus formation 10–14 days following transfection. Individual foci were selected and subcultured for further characterization.

Isolation and analysis of cytoplasmic RNA

Cells were isolated from dishes by trypsinization and pelleted by centrifugation (500 *g*, 3 min). The pellet was resuspended in lysis buffer [10 mM Tris–HCl, 1.5 mM $MgCl_2$, 140 mM NaCl, 0.5% Nonidet-40 (NP-40), pH 8.3] at a concentration of 10^7 cells/ml and lysed for 10 min at 4°C with periodic vortexing. The NP-40 lysate was centrifuged for 2 min at 14 000 *g* to pellet the nuclei, and the supernate retained. The supernate was extracted with phenol/chloroform/iso-amyl alcohol and the cytoplasmic RNA precipitated as described (Maniatis *et al.*, 1982). Cytoplasmic RNA was analysed by electrophoresis on formaldehyde-containing 1.2% agarose gels followed by capillary transfer to Biodyne A filters (Pall) as described (Rasmussen *et al.*, 1987). After transfer filters were air dried, then baked for 3 h at 68°C. For dot–blot and slot–blot analysis of RNA, 10 μg of RNA was dried by lyophilization, and resuspended in 100 μl sterile water. An equal volume of SSCF (3 vol 20 × SSC, 2 vol 37% formaldehyde) was added and the sample denatured at 70°C for 15 min. Blotting using a Mini-fold or Mini-fold II apparatus was performed according to the manufacturer's instructions (Schleicher and Schuell).

Synthesis of radiolabelled probes

Radiolabelled cDNA probes were synthesized by the oligo-labelling procedure exactly as described by Feinberg and Vogelstein (1984). The sp. act. of these probes was typically $1–2 \times 10^9$ c.p.m./μg. Before hybridization the probe was denatured by heating to 100°C for 7 min followed by rapid cooling on ice. High-specific-activity RNA probes were synthesized from a CaM-cDNA-containing pGEM-1 vector using procedures described by Promega Biotech. Each RNA synthesis reaction contained 100 μCi of SP6-grade [^{32}P]UTP (Amersham). Labelled RNA was separated from unincorporated nucleotides by repeated ethanol precipitation using 10 μg yeast rRNA as carrier. Sp. act. was always $>1 \times 10^9$ c.p.m./μg and the labelled RNA was always used within 2 days of synthesis. Before use in a hybridization, RNA probes were heated to 85°C to denature any secondary structure.

Hybridization of radiolabelled probes

Filters were pre-hybridized for 1–4 h at 42°C in 5 × SSC, 50% formamide, 5 × Denhardt's, 50 mM $NaPO_4$, pH 6.5; 1% SDS, 200 μg/ml yeast rRNA. Hybridization of radiolabelled cDNA probes was carried out for 18 h at 42°C in a solution of the same composition as that used for pre-hybridization. Probe concentration was 1×10^7 c.p.m./ml. For radiolabelled RNA probes, hybridization was carried out at 57°C for 18 h. The probe concentration was 5×10^6 c.p.m./ml. After hybridization filters were washed for 50 min at room temperature through five washes of 1 × SSC, 0.1% SDS, and for 30 min at either 55°C (for cDNA probes) or 65°C (for RNA probes) in 0.1 × SSC, 0.1% SDS to remove non-specifically bound probes. Autoradiography was carried out at −70°C using Kodak XAR-5 film and intensifying screens.

Quantitation of intracellular CaM levels

Measurement of cellular CaM levels was performed by RIA as previously described (Chafouleas *et al.*, 1979).

Flow cytometric analysis

Cells to be analysed by flow cytometry were prepared as follows. Cells were isolated by trypsinization, collected by centrifugation (500 *g*, 3 min) and the pellet resuspended in 2 ml 0.9% NaCl. The cell suspension was fixed by slow addition of 5 ml 95% ethanol while vortexing at speed 4 on a Vortex-Genie. The fixed cell suspension was allowed to stand 30 min at room temperature and subsequently stored at 4°C. Before staining, the sample was centrifuged (500 *g*, 3 min) and the fixative removed by aspiration. The pellet was disrupted and 1 ml of pepsin solution [333 μg/ml pepsin (Sigma), 55 μl 1 N HCl, 945 μl water, pH 1.5] was added. The sample was incubated for 5 min at room temperature. Pepsin was inactivated by the addition of 2 ml PS buffer (100 mM Tris–HCl, 100 mM NaCl, pH 7.4). Cells were again collected by centrifugation (500 *g*, 3 min), the buffer removed and the pellet disrupted. Cells were stained by the addition of 1 ml propidium-iodide staining solution [50 μg/ml propidium-iodide (Sigma), PS buffer]. The stained cells are stored at 4°C in the dark until ready for analysis. Twenty minutes prior to analysis, 100 μl of RNase solution [1 mg/ml RNase A (Sigma), 100 mM NaCl, Ph 8.0] was added. Flow cytometric analysis was performed using a Coulter Electronics Epic-753 flow cytometer equipped with a MDADS data analysis package.

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