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Calmodulin is involved in regulation of cell proliferation

Colin D.Rasmussen and Anthony R.Means

Department of Cell Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030, USA

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A chicken calmodulin (CaM) gene has been expressed in mouse C127 cells using a bovine papilloma virus (BPV)-based vector (BPV-CM). The vector-borne genes produce a mature mRNA of the expected size that is present on cytoplasmic polyribosomes. In clonal cell lines transformed by BPV-CM, expression of the CaM gene produced CaM levels 2- to 4-fold above those observed in cells transformed by BPV alone. Increased intracellular CaM caused a reduction of cell cycle length that is solely due to a reduction in the length of the G_1 phase. A comparison of six cell lines revealed a linear relationship between the intracellular CaM concentration and the rate of G_1 progression. These data provide the first evidence that specific elevation of CaM levels directly affects the rate of cell proliferation.

Key words: bovine papilloma virus/calmodulin/cell cycle/C127 cells/G_1 phase

Introduction

Calmodulin (CaM), the major Ca^{2+} receptor in non-muscle smooth muscle eukaryotic cells, is the mediator of many Ca^{2+}-dependent intracellular processes (for review, see Means *et al.*, 1982). However, most of the ideas about the involvement of CaM in cell function have been inferred from two lines of evidence. First, by the study of Ca^{2+}–CaM-dependent enzymes *in vitro* utilizing either cell extracts or purified protein preparations. Second, by examining the effects of pharmacological agents on cell function. Whereas these drugs inhibit the Ca^{2+}–CaM activation of enzymes *in vitro*, they are not specific in their effects on cells. A variety of studies have been interpreted to mean that CaM plays important regulatory functions in the control of cell growth and cell cycle progression. In cells (or tissues) transformed by either oncogenic viruses, chemical carcinogens or hormone treatment, the level of CaM is consistently increased (Watterson *et al.*, 1976; LaPorte *et al.*, 1980; Chafouleas *et al.*, 1981; Connor *et al.*, 1983; Veigel *et al.*, 1984; Zendequi *et al.*, 1984). This increase is the result of an enhanced rate of CaM synthesis (Chafouleas *et al.*, 1981). It has been shown both *in vitro* and *in vivo* that the depolymerizing effect of calcium on microtubule stability is mediated through CaM (Marcum *et al.*, 1978; Keith *et al.*, 1983). Previous work suggested that depolymerization of microtubules was sufficient to induce quiescent cells to enter S phase and begin DNA synthesis (Crossin and Carney, 1981). In Chinese hamster ovary (CHO) cells, intracellular CaM concentration sharply doubles as cells traverse the G_1/S boundary of the cell cycle (Chafouleas *et al.*, 1982; Sasaki and Hidaka, 1982). Experiments using the CaM naphthalenesulfonamide antagonist W-13 (Tanaka *et al.*, 1982) have suggested that this elevation of CaM levels at the G_1/S boundary is required both for re-entry of quiescent cells into the cell cycle (Chafouleas *et al.*, 1984a), and for progression through both G_1 and S in exponentially growing cells (Chafouleas *et al.*, 1982; Sasaki and Hidaka, 1982). In addition, W-13 also synergizes with bleomycin in preventing recovery from DNA damage introduced in response to the latter drug, indicating that CaM may also play a role in DNA repair (Chafouleas *et al.*, 1984b).

Both cell cycle progression and cell transformation are accompanied by characteristic alterations in cell morphology, cyclic nucleotide metabolism, metabolic rate and intracellular calcium levels, all processes considered to be influenced by CaM. In addition, transformed cells lose the requirement both for anchorage to a substrate and for the high levels of extracellular calcium required for proliferation by their non-transformed counterparts (Durkin *et al.*, 1981). Thus, many of the properties typical of transformed cells could conceivably be a result of increased intracellular CaM levels. The present study was designed to examine the role of increased intracellular CaM in the regulation of cell proliferation *in vivo* by a more direct manner than through the use of CaM antagonists. The approach taken has been to express a chicken CaM gene (Epstein *et al.*, 1987) using a bovine papilloma virus-based (BPV-based) eukaryotic expression vector. Several independently derived cell lines have been selected which stably maintain episomal copies of the BPV–CaM and have constitutively elevated CaM levels that are 2- to 4-fold above the levels observed in control cell lines transformed by a BPV vector lacking the CaM gene. The most striking consequence of increased CaM in cells expressing the BPV–CaM hybrid vector is an increase in the rate of cell proliferation due to a shortening of the cell cycle. Detailed analysis shows that this reduction in cell cycle time is entirely due to a reduction in the length of the G_1 period. In addition, the rate of progression through G_1 is positively correlated to intracellular CaM concentration. Thus, these data provide evidence that CaM has an effect on the rate of cell cycle progression.

Results

Transformation of C127 cells with a BPV–CaM expression vector

A bovine papilloma virus-based eukaryotic expression vector (BPV-CM) was constructed (Figure 1) and used to transform mouse C127 cells. BPV-CM consists of: (i) 1.3 kb of 5′-flanking sequence derived from the chicken CaM gene that contains the CaM gene promoter; (ii) a 6.6-kb CaM minigene which lacks the first two large introns (8 kb of DNA) of the CaM gene but which contains all the information necessary to encode authentic CaM (Rasmussen *et al.*, 1987) plus 3 kb of the 3′-untranslated region of the CaM gene; and (iii) the 69% transforming region of BPV-1 in a pBR322 derivative (pML2) to allow propagation in *Escherichia coli* (Sarver *et al.*, 1982). Low passage mouse C127 cells were transfected either with BPV-CM or a control plasmid pdBPV-1 and foci of transformation were selected 10–14 days following transfection. Of the foci selected, only those cell

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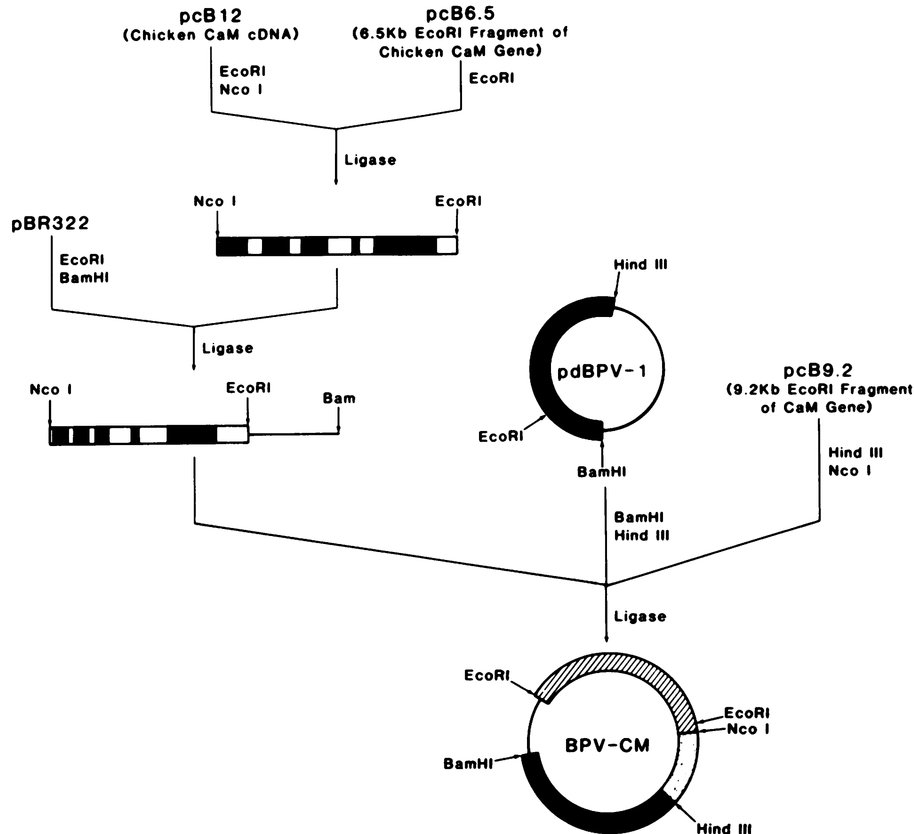


Fig. 1. Construction of a BPV−CaM hybrid expression vector. The vector BPV-CM contains a CaM minigene placed 3′ to the CaM promoter isolated from a chicken genomic clone (Epstein *et al.*, 1987). The minigene was constructed by ligating pCB6.5, a chicken genomic clone containing all but the first 30 bp of CaM coding sequence to an *NcoI*/*EcoRI* fragment of pcB12, a chicken CaM cDNA clone which contains the first 30 bp of CaM-coding sequence, in the plasmid pML2 a derivative of pBR322. The resulting minigene contains the entire CaM coding sequence but lacks the first two large introns which comprise nearly 8 kb of genomic DNA. The 9.3-kb *NcoI*/*BamHI* fragment of this intermediate plasmid was ligated to a 5.5-kb *BamHI*/*HindIII* fragment containing the 69% transforming region of BPV derived from the vector pdBPV-1 and to a 1.3-kb *HindIII*/*NcoI* fragment of the chicken CaM genomic clone pcB9.2 containing the chicken CaM promoter to produce the vector BPV-CM. The filled area corresponds to BPV, the hatched area to the CaM minigene, the speckled area to the CaM promoter and the thin line to pML2 sequences. The vector pdBPV-1 has been previously described (Sarver *et al.*, 1982) and was used as a control vector for the effect of transformation by BPV without increased CaM gene dosage.

lines which carried the BPV-CM plasmid as a nuclear episome were cloned and screened for expression of the transfected CaM gene. Of the 12 cell lines initially characterized, three had levels of cytoplasmic CaM mRNA which were 20- to 50-fold higher than those observed in BPV-transformed control cell lines (Table I). The one which displayed the greatest increase in cytoplasmic CaM mRNA, called CM-1, was selected for detailed characterization. In a parallel experiment, two focally derived cell lines which stably maintained the vector pdBPV-1 as an episome at similar copy number to the three BPV-CM lines were also selected and used as control cell lines in subsequent experiments to account for the effects of transformation by BPV alone.

CaM mRNA and CaM in transformed cells

Northern blot analysis of equal amounts of cytoplasmic RNA isolated from the BPV-1 and CM-1 cell lines is shown in Figure 2. Lane A illustrates that BPV-1 RNA, a chicken CaM cDNA (Putkey *et al.*, 1983), hybridizes predominantly to a species of 1.3 kb, as well as to minor bands of 1.7 kb and 0.9 kb. The sizes of these mRNA species are consistent with the previously reported sizes for CaM mRNAs in rat (Nojima *et al.*, 1987; Sherbeney *et al.*, 1987). In CM-1 cells, transcription of the episomally carried CaM minigene produced high levels of mRNA homologous to the chicken cDNA probe (Figure 2; lane B). A 20-fold shorter exposure of the same filter shown in lanes A and B demonstrates that the probe is recognizing a single species of 1.6 kb (Figure 2; lane C) which is identical in size to the CaM mRNA detected in chicken gizzard poly(A)$^+$ by the same probe (Figure 2; lane D). In cells treated with 5 μg/ml actinomycin D to inhibit RNA synthesis (Lilienbaum *et al.*, 1986), the vector-derived CaM mRNA has a half-life of $\sim$7 h, which is identical to that of the CaM mRNA present in cells transformed by BPV alone (Figure 3). We conclude that the CaM minigene present in the BPV-CM vector produces a transcript which is processed correctly to yield a mature cytoplasmic mRNA of the appropriate size and that has the same degree of stability as does the endogenous CaM mRNA in BPV-transformed C127 cells.

Since the BPV-CM vector efficiently and stably increased the level of CaM mRNA in CM-1 cells, we determined if there was a corresponding increase in intracellular CaM concentration. CaM was isolated from equal numbers of BPV-1 and CM-1 cells using phenyl−Sepharose chromatography and analysed by SDS−PAGE. The results are shown in Figure 4. Levels of intact CaM in CM-1 cells are increased 4- to 5-fold relative to the levels observed in BPV-1 cells as estimated by densitometric scanning of the Coomassie Blue-stained gel. Calmodulin levels in these cells were also quantitated by radioimmunoassay (RIA) as described by Chafouleas *et al.* (1979). The difference between CM-1 and BPV-1 cells found using this assay (Table I) was similar to the results obtained by SDS−PAGE.

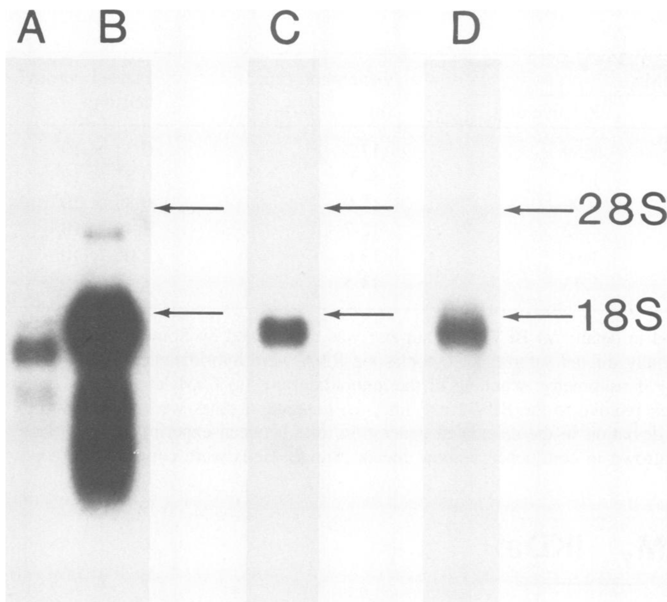


Fig. 2. Analysis of CaM mRNA in transformed C127 cells. Equal amounts (5 μg) of cytoplasmic RNA from BPV-1 (**lane A**) or CM-1 (**lane B**) cells were separated on 1.2% agarose/formaldehyde gels and blotted onto Biodyne A and hybridized to an oligo-labelled 0.3-kb *Eco*RI/*Pst*I fragment of the coding region of the chicken cDNA clone pcB12 as described in Materials and methods. Exposure time was 15 h. **Lane C** is an autoradiogram of the same filter used to produce **lanes A** and **B** , but exposure time was reduced 20-fold to 45 min. **Lane D** shows the hybridization of the probe to 5 μg of chicken gizzard poly(A)$^+$ RNA. Exposure time was 20 min. In each panel, the position of 28S and 18S rRNA observed by acridine orange staining of the gel prior to transfer is shown as a size reference.

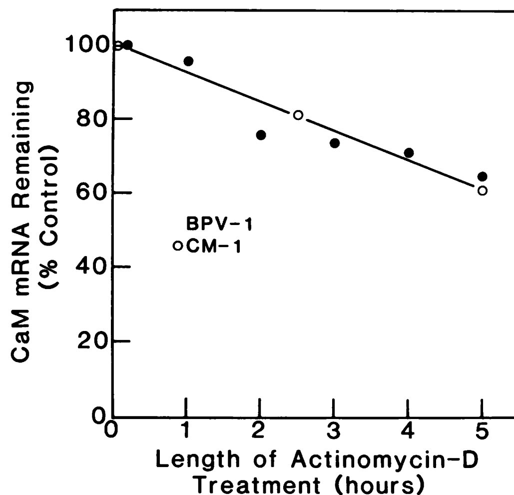


Fig. 3. Stability of CaM mRNA in BPV-1 and CM-1 cells. Expontentially growing BPV-1 (●) or CM-1 (○) cells were incubated in culture medium containing 5 μg/ml actinomycin D for the time indicated. Cells were harvested and the amount of CaM mRNA determined by Northern blot analysis. Autoradiograms were quantitated by densitometric scanning of the autoradiogram.

The CaM isolated from CM-1 cells (Figure 4; lane marked CM) is identical in size to both the endogenous mouse CaM isolated from the BPV-1 control cell line (Figure 4; lane marked BPV), and to the authentic vertebrate CaM produced by a bacterial expression vector (Figure 4, lane marked STD) (Putkey *et al.*, 1985). In addition, when the samples are electrophoresed in the presence of 5 mM Ca^{2+} (Figure 4; lanes marked Ca), CaM isolated from CM-1 cells displayed a shift in M_r identical to both mouse CaM (BPV lane) and the bacterially synthesized protein (STD lane). This shift in apparent M_r in the presence of Ca^{2+} is characteristic of many Ca^{2+}-binding proteins and indicates that the extra CaM produced by expression of the BPV-CM vector binds Ca^{2+}.

Calmodulin levels in CM-1 cells are elevated far less than are the levels of the corresponding vector-derived mRNA. This could be due either to a decreased rate of translation of the vector-derived mRNA relative to endogenous CaM mRNA, or to an increase in the rate of degradation of the protein. Since exponentially growing cells maintain intracellular protein concentrations at steady-state levels, the steady-state CaM level is given by the relationship $[CaM]_{ss} = K_s/K_d$ (Berlin and Schimke, 1965), where $[CaM]_{ss}$ is the steady-state CaM level, K_s the rate of CaM synthesis, and K_d the rate of CaM degradation. If there is no change in the rate of CaM degradation in CM-1 compared with BPV-1 cells, then the relative steady-state CaM levels in the two cell lines should be directly proportional to the rate of synthesis $SS_{(CM-1)}/SS_{(BPV-1)} = K_{s(CM-1)}/K_{s(BPV-1)}$. As an initial approach to addressing this issue, the relative rate of CaM synthesis was measured in BPV-1 and CM-1 cells and the results are presented in Figure 5. Both BPV-1 and CM-1 cells showed linear incorporation of [^{35}S]methionine over 3 h. The slope of each line gives an estimation of the rate of CaM synthesis. From these values we determined that CaM is synthesized in CM-1 cells at a rate that is 4.2 times greater than observed in BPV-1 cells. By RIA, the steady-state level of CaM in CM-1 cells was found to be 4.4 times greater than in BPV-1 cells (Table I). These data argue that the difference in the relative steady-state levels of CaM in CM-1 versus BPV-1 cells can be fully accounted for by the difference in relative rate of synthesis. We conclude, therefore, that the rate of CaM degradation is not increased in CM-1 cells and that the vector-derived mRNA must be translated less efficiently than is the endogenous CaM mRNA.

Increased CaM affects the rate of cell proliferation

Since earlier work suggested that CaM plays an important role in the regulation of cell proliferation, the growth characteristics of cells with increased CaM were determined. Five different clonal lines derived from transformation with either the pdBPV-1 control plasmid or the BPV-CM expression vector have been analysed. The BPV episome copy number, CaM mRNA and protein levels, cell density at plateau phase and cell cycle length for each line are given in Table I. Transformation of C127 cells either by pdBPV-1 or BPV-CM had effects both on cell density of cultures grown to plateau phase and on the rate of cell growth. In two independent pdBPV-1 cell lines, cell density at plateau increased 10–20% and the cell cycle was shortened by 2–2.5 h relative to non-transformed C127 cells. All three independently derived BPV-CM transformed cell lines showed a further increase in saturation density as well as an additional reduction in cell cycle length. These effects cannot simply be a consequence of BPV copy number since the BPV-1 cell line has nearly twice the number of BPV copies as the CM-1 line and four times that of the CM-2 or CM-4 lines, both of which exhibit a higher saturation density and shorter cell cycle than the BPV-1 line (Table I). These observations suggest that the differences in cell growth-related parameters observed in all three BPV-CM transformed cell lines relative to the BPV-containing control lines are likely due to elevated intracellular CaM levels.

Since increased CaM seems to shorten the cell generation time, the kinetics of cell cycle progression were determined for both

Table I. Characterization of transformed cell lines

Cell line	No. of BPV[a] episomes	CaM mRNA[b] relative amt.	Intracellular CaM[c]		Generation time[d]	Plateau density[e] cells/cm^2
			ng/10^6 cells	Relative amt.	(h)	
C127	–	1.0	240	0.59	17.3	0.73×10^5
BPV-1	150	1.5	410	1.00	14.6	0.81×10^5
BPV-2	40	1.5	370	0.90	15.0	0.85×10^5
CM-1	90	50	1810	4.42	12.7	1.49×10^5
CM-2	30	20	680	1.66	13.6	1.06×10^5
CM-4	30	20	710	1.73	13.5	1.17×10^5

Transfected cell lines which retained the input DNA as episomes were characterized in detail: (a) BPV copy number was determined by Southern hybridization as previously described (Rasmussen *et al.*, 1987); (b) dot blots of serially diluted samples of cytoplasmic RNA were hybridized to a rat CaM cDNA probe (Nojima *et al.*, 1987). Relative CaM mRNA levels were estimated by densitometric scanning of the autoradiogram; (c) CaM levels were determined by RIA as described (Chafouleas *et al.*, 1979) and the amount expressed relative to the BPV-1 cell line; (d) generation times were calculated by determining the time between mitoses in mitotically synchronized cell populations; deviation in the calculated generation time between experiments is typically ± 0.1 h; (e) cells were plated at a density of 2×10^5 cells in 60-mm dishes and grown to confluence without media change. Cells were counted twice daily until cell number no longer increased.

the CM-1 and BPV-2 cell lines. These two lines were chosen since they had the greatest difference in cell cycle length, and would therefore provide the most sensitive indicator of which cell cycle phase was being affected by increased CaM. Using the frequency of labelled mitoses method to monitor cell cycle progression in exponentially growing cells (Howard and Pelc, 1957), the relative length of each cell cycle phase was determined. Figure 6a shows that the only difference in the [^{3}H]thymidine labelling curves occurred in the region that includes those cells which resided in G_1 at the time of labelling. The length of each cell cycle component was calculated from the data in Figure 6a and is summarized in Figure 6b. The entire 2.5 h difference in cell cycle length could be accounted for by the difference in the length of G_1. Although the data shown are the results of a single experiment, identical values were obtained on four separate occasions. These data suggest that increased CaM levels affect the rate at which cells proliferate by shortening the G_1 phase.

In order to confirm and extend this result, the length of G_1 was directly determined in all cell lines by an alternative method. First, synchronous populations of cells were obtained by the mitotic shake-off procedure (Terasima and Tolmach, 1961), and re-plated into culture media containing [^{3}H]thymidine. At various times after re-plating, cell samples were isolated and the net amount of [^{3}H]thymidine incorporated into DNA determined. The time at which cells begin to incorporate [^{3}H]thymidine at a high rate marks the onset of S-phase and the interval from mitosis to S is a direct measurement of the duration of G_1. Figure 7a shows that the length of G_1 is reduced in all three BPV-CM cell lines as compared with either of the BPV-transformed controls or untransformed C127 cells. In each case the differences observed in cell cycle length is completely accounted for by the differences in the measured length of the G_1 period (Table II). Thus, without exception, shortening of the cell cycle occurs via a reduction in the length of G_1. When the data in Figure 7a are expressed graphically with the reciprocal of G_1 length plotted as a function of intracellular calmodulin concentration on a logarithmic scale, a linear relationship is observed (Figure 7b). This result shows that there is a strong positive correlation between intracellular CaM levels and G_1 length ($r = 0.994$; $P \ll 0.001$; df = 4). Taken together thse data support our earlier hypothesis that CaM plays a central role in controlling the rate of cell proliferation by regulating the rate at which cells progress through G_1 and enter S phase.

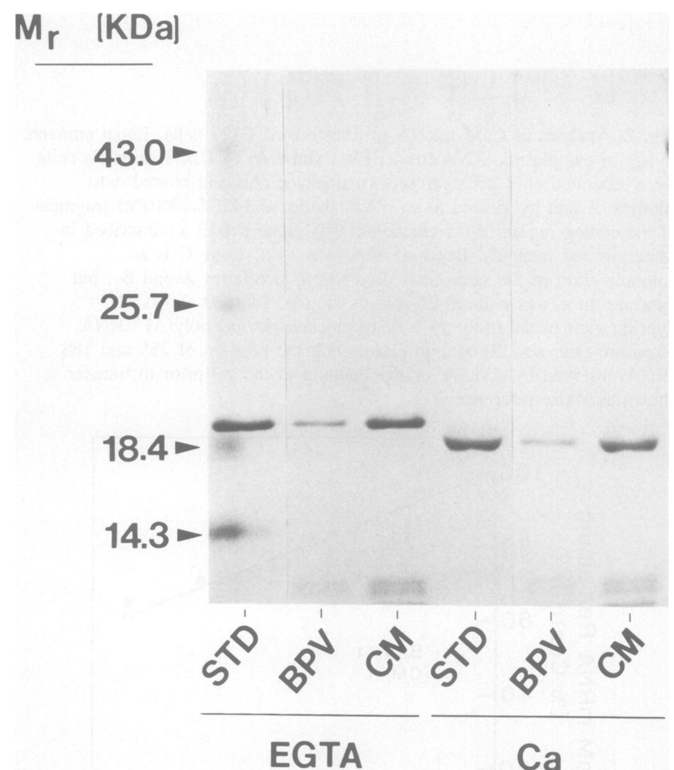


Fig. 4. CaM in transformed cell lines. CaM from equal numbers of BPV-1 (BPV) and CM-1 (CM) cells was isolated and electrophoresed as described in Materials and methods. The lane marked STD contained 2 μg of purified chicken CaM produced in a bacterial expression vector and isolated as previously described (Putkey *et al.*, 1985). Prior to solubilization, either 5 mM EGTA (lanes marked EGTA) or 5 mM Ca^{2+} (lanes marked Ca) was added to the sample.

Discussion

The present study represents an initial attempt to alter cell function by constitutively increasing intracellular CaM levels via the expression of a transfected CaM gene. To accomplish this, a chicken CaM minigene, regulated by its own promoter, has been expressed in mouse C127 cells in a BPV-based eukaryotic expression vector. This virus and mouse cell line were chosen for several reasons. First, the BPV genome is stably maintained in C127 cells over many cell generations at high copy numbers. Thus, high levels of expression of the vector-borne gene are possible when a suitable promoter is used. Second, transformed C127 cells can be selected by focus formation exactly as are cells derived using a selectable marker (Law *et al.*, 1983). Third, BPV

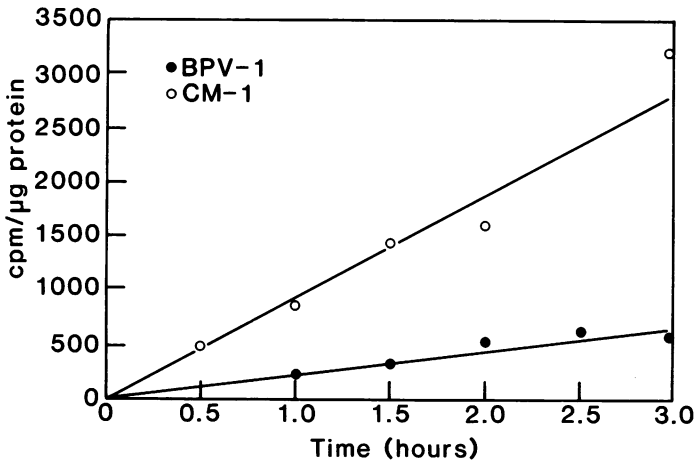
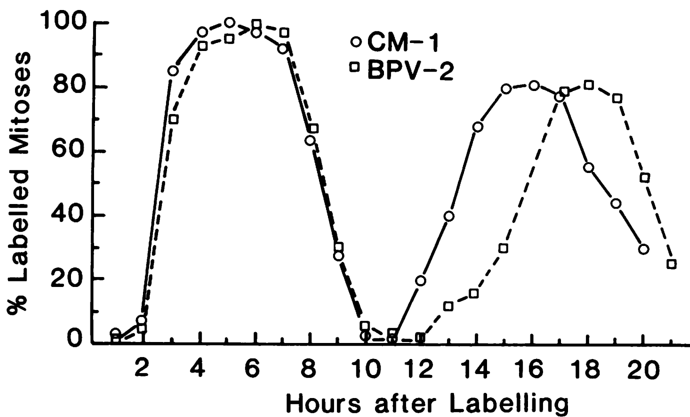


Fig. 5. Estimated rate of calmodulin synthesis in BPV-1 and CM-1 cells. The amount of [^{35}S]methionine incorporated into immunoprecipitable CaM in exponentially growing BPV-1 (filled circles) and CM-1 (open circles) was determined as described by Chafouleas *et al.* (1981). The relative rate of synthesis is determined from the slope of each line.



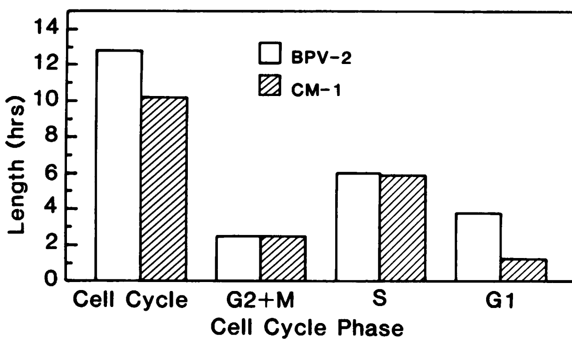
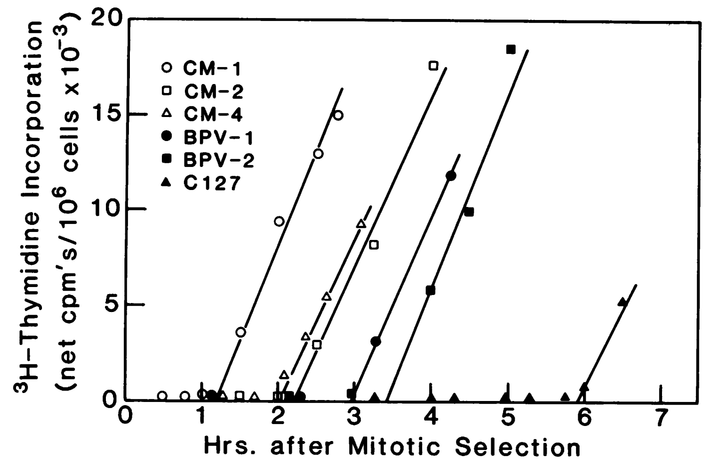


Fig. 6. Cell cycle analysis of BPV-transformed cell lines. (**a**) The frequency of labelled mitoses in exponentially growing BPV-2 and CM-1 cells was determined and analysed as described in Materials and methods. In analysing the cell cycle kinetics from these two cell lines, the superimposition of the first wave of labelled mitoses indicates that G_2, S and M phases are of similar lengths in the two cell types. The delay of occurrence of the second wave of labelled mitoses in BPV-2 cells indicates that BPV-2 cells have a longer G_1 phase than CM-1. (**b**) Summary of data in **a**. The cell cycle phase lengths were calculated as described in Materials and methods. The only cell cycle phase which differs in length by this analysis is G_1. The duration of all other cell cycle phases was unaffected.



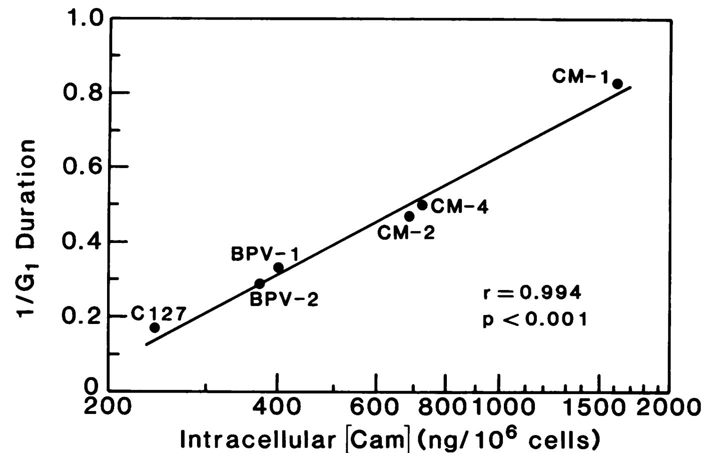


Fig. 7. G_1 duration in transformed C127 cells. (**a**) Net incorporation of [^{3}H]thymidine in mitotically synchronized cells. The duration of G_1 is calculated as the intercept of the linear portion of the labelling curve with the *x*-axis. (**b**) Relationship between CaM levels and entry into S-phase. The data obtained from **a** were plotted as the reciprocal of G_1 duration (analogous to rate of entry into S) as a function of [CaM]. The correlation coefficient **r** = 0.994 is significant at $P \lll 0.001$.

Table II. Relationship between differences in generation time and G_1 duration

Cell line	Generation time (h)	Difference (h)	G_1 duration (h)	Difference (h)
CM-1	12.7	–	1.2	–
CM-2	13.6	0.9	2.2	1.0
CM-4	13.5	0.8	2.0	0.8
BPV-1	14.6	1.9	3.0	1.8
BPV-2	15.0	2.3	3.4	2.2
C127	17.3	4.6	5.9	4.7

Generation times were calculated as described in Table I. G_1 durations are derived from the data presented in Figure 7 as described in Materials and methods. Differences in both generation time and G_1 duration are expressed relative to the CM-1 cell line.

vectors are usually maintained as nuclear episomes in the C127 cells (Law *et al.*, 1981). The lack of random integration of BPV-linked sequences into the host genome precludes the possibility of altering (either positively or negatively) a host gene required for normal cell function. In this study, only cells which contained unrearranged, episomal copies of the input DNA were retained for subsequent analysis. Therefore, it is unlikely that any of the changes observed can be attributed to alterations in the host genome as a consequence of the transfection procedure. This is in contrast to other commonly used vectors, such as those based on retroviruses (van der Putten *et al.*, 1985) for which integration

into the host genome is a prerequisite for stable maintenance.

In the three BPV-CM transformed cell lines examined, CaM mRNA levels were elevated 20- to 50-fold, while CaM levels were increased 2- to 4-fold above the BPV-transformed controls. Characterization of the vector-derived mRNA showed that it appeared in the cytoplasm as a single mature species of the expected size. The observation that CaM does not increase concomitantly with CaM mRNA in BPV-CM transformed cell lines suggests that either the vector-derived CaM mRNA is translated inefficiently, or CaM is turned over more rapidly. Since the relative steady-state CaM levels in BPV-1 and CM-1 cells can be accounted for by the estimated relative rates of CaM synthesis, it is likely that the vector-derived mRNA is translated at a lower rate than the endogenous CaM mRNA.

The most readily apparent phenotypic effect of elevating CaM concentration is a decrease in the length of the cell cycle which we have shown is due to a reduction in the length of the G_1 phase. The observation that the G_1 phase of the cell cycle was affected was not unexpected. It is well established that G_1 is the most variable portion of the cell cycle and that changes in cell cycle length are achieved by varying the length of G_1 (Zetterberg and Larsson, 1984). Previous studies suggested that CaM is involved in G_1 progression (Chafouleas *et al.*, 1982, 1984a). Several additional studies have demonstrated the anti-proliferative effects of CaM antagonists, which have been shown to reversibly block cell cycle progression in G_1 and early S (Goyns and Hopkins, 1981; Hait *et al.*, 1985; Lee and Hait, 1985; Suzuki *et al.*, 1986; Rainteau *et al.*, 1987). The results in this study demonstrate that a specific elevation of CaM levels affects the rate of cell proliferation by increasing the rate at which cells progress through G_1.

Because of the ability of CaM to potentially interact with a large number of intracellular proteins it is likely that the observed effects on cell cycle progression are mediated through a set of interrelated pathways rather than by acting upon a single target enzyme. It is unlikely that the effect of CaM on cell cycle progression is primarily via its putative role in DNA repair although similar molecular mechanisms may be common to the two processes. It is also unlikely that the effect is due to stimulation of any single CaM-dependent enzyme described so far. However, several processes which are correlated with cell cycle progression and which might be affected by CaM do occur during G_1. First, it has been well established by *in vitro* studies that CaM regulates its target enzymes only when bound to calcium. Calcium has long been implicated as a regulator of cell proliferation in several systems (Whitfield *et al.*, 1976). Cells transformed by oncogenic viruses are commonly able to proliferate in Ca^{2+}-deficient medium that prevents the growth of their non-transformed counterparts (Boynton *et al.*, 1977; Durking *et al.*, 1981). It appears that the requirement for high levels of Ca^{2+} occurs during the latter part of G_1 in both exponentially growing cells and cells stimulated to proliferate by the addition of serum (Hazelton *et al.*, 1979). We are currently examining what effect elevation of CaM has on the Ca^{2+} requirement for proliferation of CM-1 cells.

Second, previous studies have suggested that the cytoplasmic microtubule complex acts as a signal transduction mechanism in the regulation of DNA synthesis. Microtubule depolymerizing drugs have been shown to stimulate DNA synthesis in quiescent cell cultures, while the microtubule-stabilizing drug Taxol inhibits the effects of these drugs (Crossin and Carney, 1981). The $Ca^{2+}-$CaM complex has been shown both *in vitro* (Marcum *et al.*, 1978; Job *et al.*, 1980) and by microinjection *in vivo* (Keith *et al.*, 1983) to promote disassembly of microtubules, possibly by CaM-dependent protein kinase phosphorylation of tubulin or microtubule-associated proteins (Sahyoun *et al.*, 1986; Wandosell *et al.*, 1986). Increased CaM would therefore be expected to increase the level of unpolymerized tubulin in cells. It has been previously shown that increased tubulin monomer causes a reduction in the levels of tubulin mRNA through changes in mRNA stability (Cleveland *et al.*, 1981). Preliminary results indicate that β-tubulin mRNA levels in CM-1 cells are decreased >2-fold as compared with BPV-1 cells (data not shown). The role of CaM in regulating the dynamic state of the cytoskeleton may be an important mechanism controlling the onset of DNA synthesis.

Third, calmodulin is only one of a number of gene products whose levels are regulated in a cell cycle-dependent manner during G_1 and early S phase (Chafouleas *et al.*, 1982). Others, mostly involved in events related to DNA synthesis, are proteins such as transferrin (Miskimins *et al.*, 1986), thymidine kinase (Liu *et al.*, 1985), histone H1 kinase (Woodford and Pardee, 1986), dihydrofolate reductase (Farnham and Schimke, 1986) and a protein previously named cyclin (Prelich *et al.*, 1987a) which has been recently cloned (Almendral *et al.*, 1987) and shown to stimulate DNA polymerase delta activity (Prelich *et al.*, 1987b). In addition, several cDNAs which are specifically expressed during G_1 have been cloned (Hirschorn *et al.*, 1984), and a gene necessary for G_1 progression in baby hamster kidney cells has also been recently isolated (Greco *et al.*, 1987). It is possible that one or more of these proteins may be regulated by calmodulin.

Finally, calmodulin genes in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have recently been isolated and shown to be essential for cell growth (Davis *et al.*, 1986; Takeda and Yamamoto, 1987). The effect of CaM gene disruption in haploid *S.pombe* spores suggests that the amount of CaM per cell is near the minimum needed for proliferation. This is consistent with our earlier suggestion that CaM may function as a regulatory factor for cell cycle progression (Chafouleas *et al.*, 1982) and the results presented in this study which demonstrate a correlation between CaM concentration and the rate of progression through G_1. Cell cycle genetics have been extensively studied in yeast. It is known that the rate limiting step in cell proliferation is controlled by the execution of an event in G_1 termed *start*. The genetic locus responsible for *start* has been cloned in both *S.cerevisiae* and *S.pombe* and in both cases the product of the *start* gene is a protein kinase (Reed *et al.*, 1985; Simanis and Nurse, 1986). Recently, the human homologue of *start* was cloned by complementation of conditional *S.pombe start* mutants (Lee and Nurse, 1987). The predicted sequence of the human equivalent to *start* indicates that this gene also encodes a protein kinase. Thus, not only does the signal to proliferate seem intrinsically tied to the activity of a protein kinase but it appears that this activity is highly conserved among eukaryotes. Protein phosphorylation is an important feature of the cell cycle. A large number of proteins are known to be phosphorylated and dephosphorylated in a cell cycle-dependent fashion. These include histones, ribosomal proteins, elements of the cytoskeleton and several as yet unidentified proteins (Thomas *et al.*, 1982; Westwood *et al.*, 1985; Morla and Wang, 1986; Tripp *et al.*, 1986). Because of its ability to directly stimulate a large number of protein kinases and at least one phosphatase CaM could potentially regulate cell proliferation by modulating the phosphorylation of key cellular proteins.

Materials and methods

Cell culture

Mouse C127 cells are a non-transformed line derived from a mouse mammary tumor (Lowy *et al.*, 1978) and were obtained from Dr Dean Hamer of the NIH. The cells are routinely cultured in Dulbecco's modified Eagle's medium/high glucose (DMEM) containing 10% fetal calf serum (FCS) (both from Gibco) at 37°C in a humidified, 95% air:5% CO_2 atmosphere, and passaged when the cells were 70 to 80% confluent. Cells are maintained in T-flasks (75 cm^2; Falcon).

Transfection of cells and selection of transformed foci

Early passage C127 cells were transfected with either pdBPV-1 or BPV-CM DNA as previously described (Rasmussen *et al.*, 1987). Transformed cells were selected on the basis of focus formation. Individual foci of sufficient size (5 mm) were selected and subcultured for further characterization.

Preparation of cytoplasmic RNA, gel electrophoresis and Northern transfer

Cells were isolated from dishes by trypsinization and pelleted by centrifugation (500 *g*; 5 min). The pellet was resuspended in Nonidet P-40 (NP-40) lysis buffer (10 mM Tris−HCl/pH 8.3; 1.5 mM $MgCl_2$; 140 mM NaCl; 0.5% NP-40) at a concentration of 10^7 cells/ml. Cells were lysed for 10 min at 4°C with periodic vortexing. The NP-40 lysate was centrifuged to pellet nuclei (12 000 *g*; 2 min). Total cytoplasmic RNA was prepared from the supernatant fluid by extraction with phenol/chloroform and precipitation of the RNA from ethanol as described (Maniatis *et al.*, 1982). Cytoplasmic RNA was analysed by electrophoresis on formaldehyde-containing 1.2% agarose gels followed by transfer to Biodyne A filters (Pall) as described previously (Rasmussen *et al.*, 1987). After transfer filters were air-dried and baked 3 h at 68°C.

Labelling DNA and hybridization conditions

^{32}P-Labelled DNA probes were prepared by the oligo-labelling method (Feinberg and Vogelstein, 1984) and had specific activities which were typically $1-2 \times 10^9$ c.p.m./μg. Filters were pre-hybridized for 1−2 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 labelled probe to Northern transfers was carried out at 42°C for 18−24 h in a solution of the same composition as was used for pre-hybridization, with labelled probe added at a concentration of 1×10^7 c.p.m./ml of hybridization solution. Blots were washed for 1 h at room temperature through five changes of 1 × SSC/0.1% SDS, and for 30 min at 55°C in 0.1 × SSC/0.1% SDS to remove non-specifically bound probe. Autoradiography was carried out at −70°C using Kodak X-AR film with intensifying screens.

Analysis of CaM protein from transfected cells

Measurement of cellular CaM levels was accomplished by RIA as previously described (Chafouleas *et al.*, 1979). The method for the isolation of CaM from cultured cells by phenyl−Sepharose chromatography has also been described (Rasmussen *et al.*, 1987). Cells were removed from dishes by scraping, resuspended in Buffer A (50 mM Tris−HCl/pH 7.5; 5 mM $CaCl_2$) and sonicated for 20 s at low power using a Biosonik IV sonicator. The sonicated sample was heat-treated for 3 min at 90 to 95°C, cooled on ice and centrifuged for 5 min in a microcentrifuge to pellet heat-denatured proteins. The supernate was applied to phenyl−Sepharose previously equilibrated in buffer, washed with Buffer A containing 300 mM NaCl and CaM eluted with 50 mM Tris−HCl/pH 7.5; 2 mM EDTA. Eluted protein was concentrated by ultrafiltration using Centricon 10 microconcentrators (Amicon), and protein from equal numbers of cells analysed on 15% SDS−polyacrylamide gels (Laemmli, 1970).

Estimation of calmodulin synthesis rate

The relative rate of CaM synthesis in exponentially growing BPV-1 and CM-1 cells was determined as previously described (Chafouleas *et al.*, 1981). Exponentially growing cell cultures were pre-treated by incubation in methionine-free medium to deplete the intracellular methionine pool and subsequently cultured in methionine-free medium containing 100 μCi/ml of [^{35}S]methionine. At timed intervals cells were isolated and the amount of [^{35}S]methionine incorporated into total protein and calmodulin determined as previously described (Chafouleas *et al.*, 1981).

Cell cycle kinetics

The lengths of mitosis (M), G_1, S and G_2 were determined as described by Bolton and Barranco (1975). Exponentially growing cells were pulse-labelled with 1 μCi/ml [^{3}H]thymidine (1.9 Ci/mmol; Schwarz-Mann) for 15 min. The labelling medium was removed, cells rinsed with Hank's balanced salt solution (HBSS; Gibco), and fresh growth medium added. At hourly intervals cells were isolated with 0.1% trypsin, washed with HBSS and fixed in methanol:acetic acid (3:1) for 2 h at −20°C. Cells were squashed onto clean microscope slides and processed for autoradiography as previously described (Chafouleas *et al.*, 1982). After developing, the percentage of labelled mitoses at each time point following the [^{3}H]thymidine pulse was determined, and the duration of each cell cycle phase calculated (Howard and Pelc, 1957).

Synchronization of cells by mitotic selection

Synchronous samples of mitotic cells were obtained without the use of drug treatment, by the mitotic shake procedure (Terasima and Tolmach, 1961). Cells were selectively shaken from a monolayer of exponentially growing cells and retained in M phase by placement in medium at 4°C. This procedure results in starting populations of cells which contain >95% mitotic cells. The cells were released into the cell cycle by replating into 60-mm dishes containing culture medium (DMEM + 10% FCS) at 37°C. Cell cycle progression was monitored by incorporation of [^{3}H]thymidine into TCA-precipitable DNA as previously described (Chafouleas *et al.*, 1982). The length of G_1 was calculated by extrapolation of the labelling curve to zero incorporation.

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