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Targeted neutralization of calmodulin in the nucleus blocks DNA synthesis and cell cycle progression

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Abstract

Calmodulin (CaM) is a major intracellular calcium binding protein which has been implicated in the regulation of cell proliferation. Previous studies using chemically synthesized CaM antagonists and anti-sense RNA indicated that CaM is important for initiation of DNA synthesis and cell cycle progression. However, these methods reduce total intracellular CaM and globally interfering with all the CaM-dependent processes. In order to explore the function of nuclear CaM during the cell cycle, a CaM inhibitor peptide was targeted to the nucleus of intact mammalian cells. Cell progression through S-phase was assessed by incorporation of the thymidine analogue, BrdU. Cells were transfected for 48 h with either the CaM inhibitor peptide gene or the control plasmid prior to analysis. Approx. 70% of the control cells incorporated BrdU. In striking contrast, double immunofluorescent labeling demonstrated that none of the cells expressing the CaM inhibitor peptide entered S-phase. This result indicates that neutralization of nuclear CaM by targeted expression of a CaM inhibitor peptide blocks DNA synthesis and cell cycle progression.

Keywords: Calmodulin inhibitor peptide; Nucleus; Cell cycle

1. Introduction

Calmodulin (CaM) is a major intracellular calcium binding protein [1]. CaM mediates the Ca^{2+} signal through direct interactions with its target proteins. A common feature of CaM binding proteins is that they contain an endogenous autoregulatory domain [2]. This domain is composed of a pseudosubstrate region which overlaps the CaM binding site of the enzyme. When intracellular Ca^{2+} is at resting levels, the pseudosubstrate region binds to the catalytic site of the enzyme, suppressing activity. Transient increases of intracellular free Ca^{2+} cause CaM to undergo a conformational change; Ca^{2+}/CaM then binds its target site on the enzyme, dislocating the autoinhibitory domain and thereby activating the enzyme (for review, see Ref. [3]). The CaM binding sites of target enzymes are ideal CaM inhibitors.

CaM has been suggested to be involved in the regulation of cell proliferation [4,5]. Chafouleas et al. [6] reported that total cellular CaM increases at late G1 in CHO-K1 cells. Rasmussen and Means [7] found that constitutive elevation of intracellular CaM levels shortened the cell cycle time mainly by reducing the G1 phase. In contrast, CaM antagonist studies, such as W7 and W13, demonstrated that the cell cycle was blocked at the G1/S boundary, and DNA synthesis was inhibited [6,8]. The major disadvantage of these pharmacological agents is their non-specificity. These hydrophobic compounds interact with other intracellular proteins, such as protein kinase C, oncomodulin, troponin C, and membrane receptors, in addition to CaM [9]. Rasmussen and Means [10] found that transient expression of CaM anti-sense RNA in mouse C127 cells caused a temporary cell cycle arrest at G1. Reddy et al. [11] reported that DNA synthesis was blocked when monoclonal antibody against CaM was applied to permeabilized CHO cells. In these studies, the total cellular CaM level was altered and all CaM-dependent processes were affected.

Regulation of cell growth and proliferation is a complex process involving signalling between the plasma membrane, nuclear and cytoplasm. Many cell cycle regulatory events, such as DNA synthesis, occur in the nucleus. CaM has been shown to be a dynamic component of this

Abbreviations: CaM, calmodulin; CaMBP, calmodulin binding peptide; MLCK, myosin light chain kinase; BrdU, 5-bromo-2′-deoxyuridine.

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organelle. Gough and Taylor [12] reported that the concentration of CaM can be significantly higher in the nucleus than in the cytosol. In addition, Pruschy et al. [13] found that CaM does not freely diffuse into the nucleus; it is imported via a facilitated transport mechanism. The precise role of CaM in regulating nuclear functions remains undefined. In order to explore the function of nuclear CaM during the cell cycle, a potent CaM inhibitor peptide containing nuclear localization sequences was expressed to specifically neutralize nuclear CaM.

2. Materials and methods

2.1. Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc.) containing 10% fetal bovine serum at 37°C in a 5% CO_2 humidified incubator. The mammalian expression construct pSVL-$(CaMBP)_4$ [14] was transiently transfected into COS-7 cells using a modified calcium precipitation method [15]. Recombinant DNA (30 μg) was mixed with 0.25 M $CaCl_2$, precipitated with BES buffer (pH 6.95), and evenly added to COS cells plated at a concentration of 5×10^4 cells/ml. After 20 h of incubation, cells were washed with PBS and fresh medium was added. Parental plasmid pSVL was used as the control.

2.2. Immunostaining

Transfected cells were grown on 22 × 22 mm coverslips. At 47 h after transfection, cells reached approx. 60% confluency and were incubated with 5-bromo-2′-deoxyuridine (BrdU) labeling medium with the final concentration of 10 μmol/l BrdU (Boehringer Mannheim Co.) for 1 h. Cells on coverslips were washed in PBS and fixed in 70% ethanol (in 50 mmol/l glycine buffer, pH 2.0) for 20 min at −20°C. Fixed cells were incubated with monoclonal antibody against BrdU at 37°C for 30 min, washed

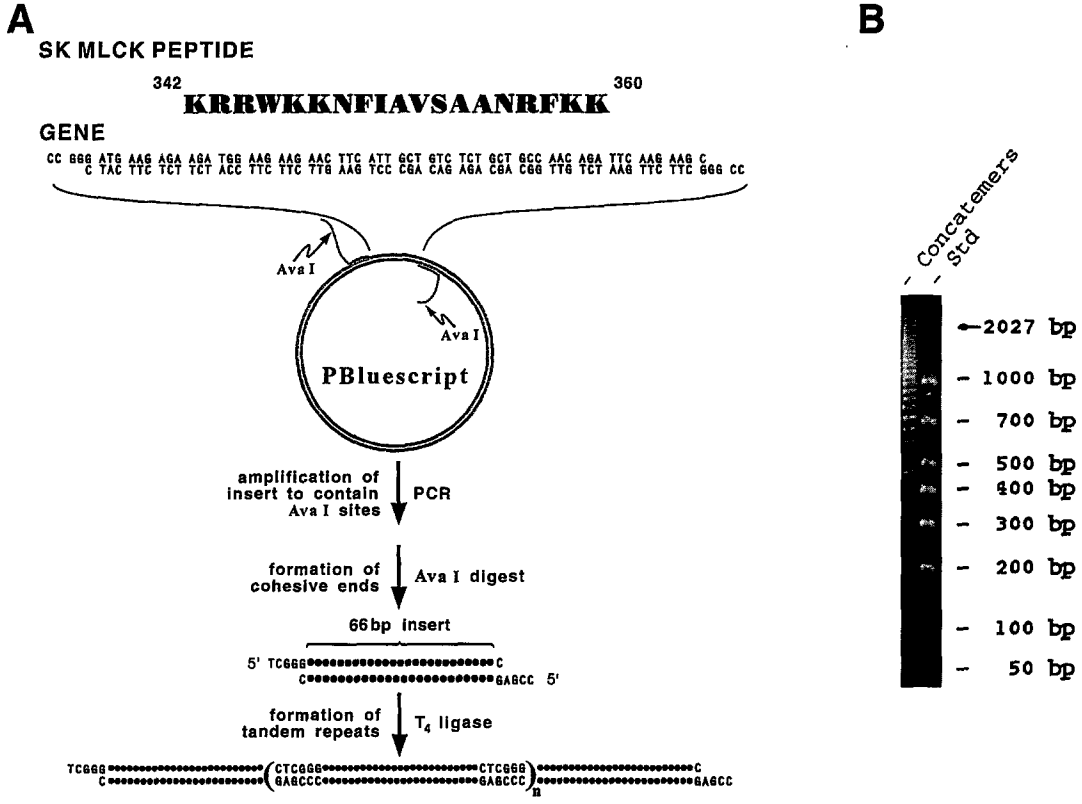

Fig. 1. Design and construction of calmodulin inhibitor peptide concatemer genes. (A) Two 72 bp oligonucleotides coding for the skMLCK CaM binding site and its complimentary sequence, each containing *Xma*I restriction enzyme overhangs, were synthesized in the DNA Core Facility at University of Cincinnati. Equimolar amounts of each synthetic oligonucleotide (1 μg) were annealed by denaturing at 95°C for 5 min followed by slow cooling to room temperature. The double-stranded *Xma*I-*Xma*I fragment was cloned into the *Xma*I site of vector pBluescript SK II (Stratagene) using T4 DNA ligase (USB). The appropriate orientation and sequence of the synthetic peptide gene (CaMBP) was confirmed by Sanger's dideoxy DNA sequencing method (USB). Two primers, (upstream 5′-CGTA*CTCGGG*ATGAAGAGAAGATGG-3′ and downstream 5′-ACTA*CCCGAG*CTTCTTGAATCTGTT-3′) were used to introduce an *Ava*I restriction enzyme site (underlined) at each end of the cloned CaMBP monomer gene. Polymerase chain reaction (PCR) was performed at 94°C for 30 s, 57°C for 30 s and 72°C for 1 min for 30 cycles (Thermolyne) using Vent polymerase (NEB). (B) PCR products (10 μg) were purified by phenol/chloroform extraction, digested with *Ava*I restriction enzyme and self-ligated at 16°C overnight using T4 DNA ligase (USB). The ligation mixture was analyzed in a 2% agarose gel.

and treated with fluorescein-conjugated sheep anti-mouse-IgG. Cells were then immunostained with affinity-purified rabbit-anti-MLCK peptide antibody [14] visualized with CY3-conjugated goat anti-rabbit IgG (Jackson Laboratories). The coverslips were then mounted and photographed using Kodak Elite 200 Ektachrome film with a Nikon Microflex UFX exposure attachment mounted on a Nikon Optiphot epifluorescence microscope with the appropriate Nikon fluorescence filter cubes.

3. Results and discussion

The CaM binding region of rabbit skeletal muscle myosin light chain kinase (skMLCK) was selected to be expressed as a specific CaM inhibitor. This 19 amino acid peptide has a high binding affinity for calmodulin ($K_d = 1$ nM) [16]. In vitro studies have shown that the synthetic MLCK peptide binds CaM in a Ca^{2+}-dependent manner and is a competitive inhibitor of several CaM-dependent enzymes [17]. A 72 base pair synthetic gene encoding the 19 amino acid skMLCK calmodulin binding site was designed and synthesized (Fig. 1A). This synthetic gene contained the *Xma*I restriction enzyme site 'CCCGGG' to facilitate cloning into pBluescript SK.

The CaM binding peptide gene was constructed as tandem repeats for several reasons. First, since CaM is an abundant protein in cells, it was important to maximize the CaM binding capacity of each transcript. Second, cells do not utilize small genes; larger precursors are synthesized and processed post-translationally. In addition, small peptides are unstable in the cytosol [18]. The multiple joined segments of the CaM binding peptide would prevent protein product degradation. Finally, tandem repeats of the CaM binding peptide create several nuclear localization signals, ensuring that the inhibitor peptide is properly targeted and concentrated within the nucleus.

The strategy used to construct the concatemer genes included PCR amplification to incorporate *Ava*I restriction enzyme sites (CTCGGG) at the ends of each monomer gene (Fig. 1A). Following *Ava*I digestion, the 66 bp monomer gene self-ligated into head-to-tail tandem repeats. This procedure produced a complex mixture of concatemers ranging from 2 to more than 40 monomer units (Fig. 1B). The ligation mixture was blunt-ended and cloned into the *Sma*I site of pBluescript SK. Clones containing 2, 3, 4, 6, 8, and 14 repeats of the CaM binding peptide gene were characterized (Fig. 2).

We elected to use a four-repeat concatemer gene since the protein product would be approx. 12 kDa and would contain multiple nuclear localization signals (NLS), including K-K/R-X-K/R and the KK.....KK bipartite motif (Fig. 3A). Many nuclear proteins such as the SV40 T antigen, DNA polymerase α and transcription factors contain such consensus sequences for nuclear targeting [19,20]. The amino acids Leu, Gly, and Met, act as a spacer

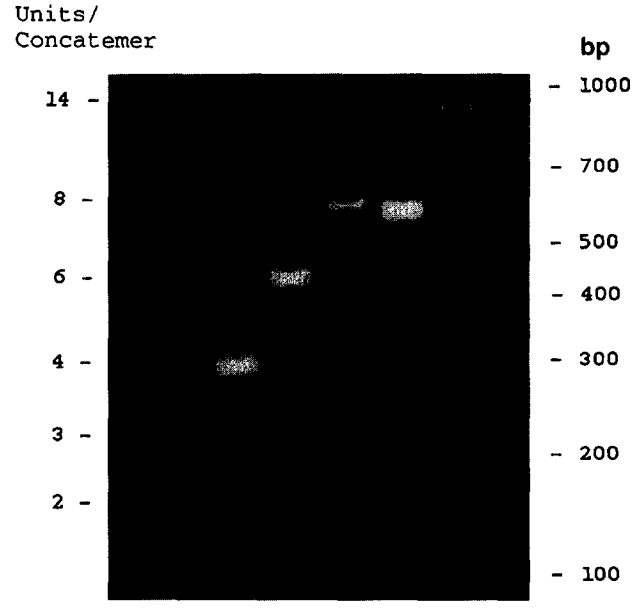

Fig. 2. Electrophorestic analysis of cloned concatemer calmodulin inhibitor peptide genes. The self-ligated concatemer mixture was blunt-ended using Klenow enzyme (Life Technologies Inc.) and cloned into the *Sma*I site of pBluescript SK. Recombinant DNA was transformed into XL1-Blue competent cells (Stratagene). The cloned genes were released from the vector by *Not*I and *Eco*RI digestion and separated on a 2% agarose gel. Concatemer genes containing 2, 3, 4, 6, 8, and 14 repeats were cloned.

between each CaM binding unit. The concatemer CaM inhibitor peptide is capable of binding multiple CaM molecules, thus preventing activation of CaM target proteins in the nucleus (Fig. 3B,C).

Wang et al. [14] found that expression of this CaM inhibitor peptide caused the formation of irregular nuclei. The cell cycle check point that was blocked by the CaM inhibitor peptide was not determined. Incorporation of BrdU, a thymidine analogue, into DNA is an indicator of DNA synthesis. This activity during the S-phase of the cell cycle is required for cell proliferation. COS-7 cells were transfected with the recombinant concatemer $(CaMBP)_4$ gene (Fig. 4A). After 48 h, cells were pulsed with BrdU for 1 h, fixed and immunostained with monoclonal anti-BrdU antibody to determine progression into the S-phase. Immunostaining with affinity-purified anti-MLCK peptide antibody indicated that approx. 40–50% of the cells were transfected with recombinant $(CaMBP)_4$ (Fig. 4B). Expressed CaM inhibitor peptides were localized within the nucleus. Double immunostaining of transfected cells indicated that cells which expressed the CaM inhibitor peptide did not incorporate BrdU (Fig. 4C,D). In contrast, approx. 70% of cells transfected with a control plasmid incorporated BrdU (Fig. 4E). These results demonstrated that DNA synthesis did not occur in cells which expressed the CaM inhibitor peptide.

Neutralization of nuclear CaM with a CaM binding peptide prevented DNA synthesis. This result indicates that

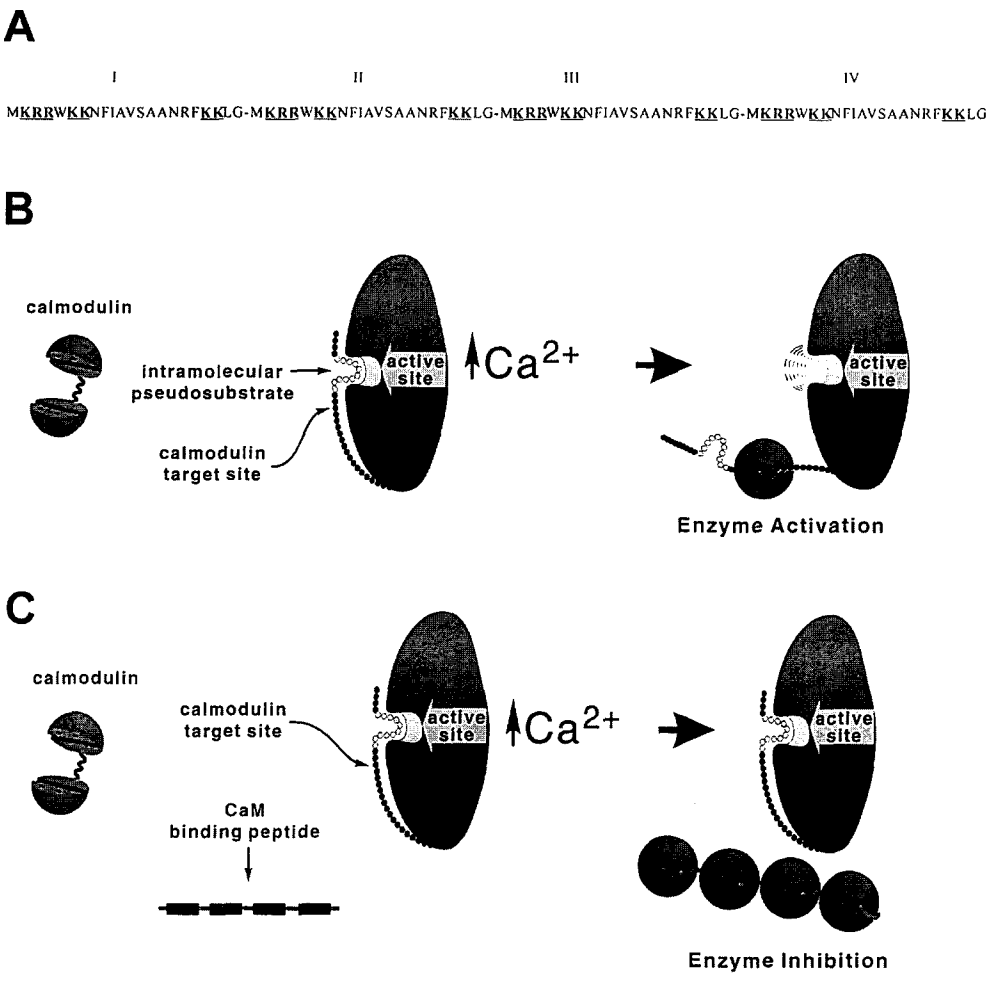

Fig. 3. Neutralization of nuclear calmodulin by targeted expression of calmodulin inhibitor peptide. (A) The derived amino acid sequence of the four repeat CaM inhibitor peptide gene contains multiple nuclear localization signals (underlined). (B) Activation of a Ca^{2+}/CaM-dependent binding protein. (C) Inactivation of a Ca^{2+}/CaM target protein by neutralization of CaM function with CaM inhibitor peptide.

nuclear CaM plays an important role in progression of the cell cycle into S-phase. The activity of DNA polymerase α, which is critical in DNA replication, has been reported to be inhibited by the CaM antagonists W13 and TFP, thus blocking the cell cycle at G1/S boundary of NRK cells [21]. The membrane permeable CaM kinase II inhibitor, KN-93, arrested Hela cells and NIH 3T3 cells at G1/S [22,23]. None of the DNA replicative enzymes, however, have been shown to be directly regulated by Ca^{2+}/CaM.

A dominant-negative strategy to design peptides which inhibit target proteins within specific cellular compartments allows for molecular dissection of cellular function. We have applied this approach to evaluate the role of nuclear CaM during the cell cycle. Selective inhibition of individual CaM target enzymes will further delineate the mechanism of cell cycle regulation.

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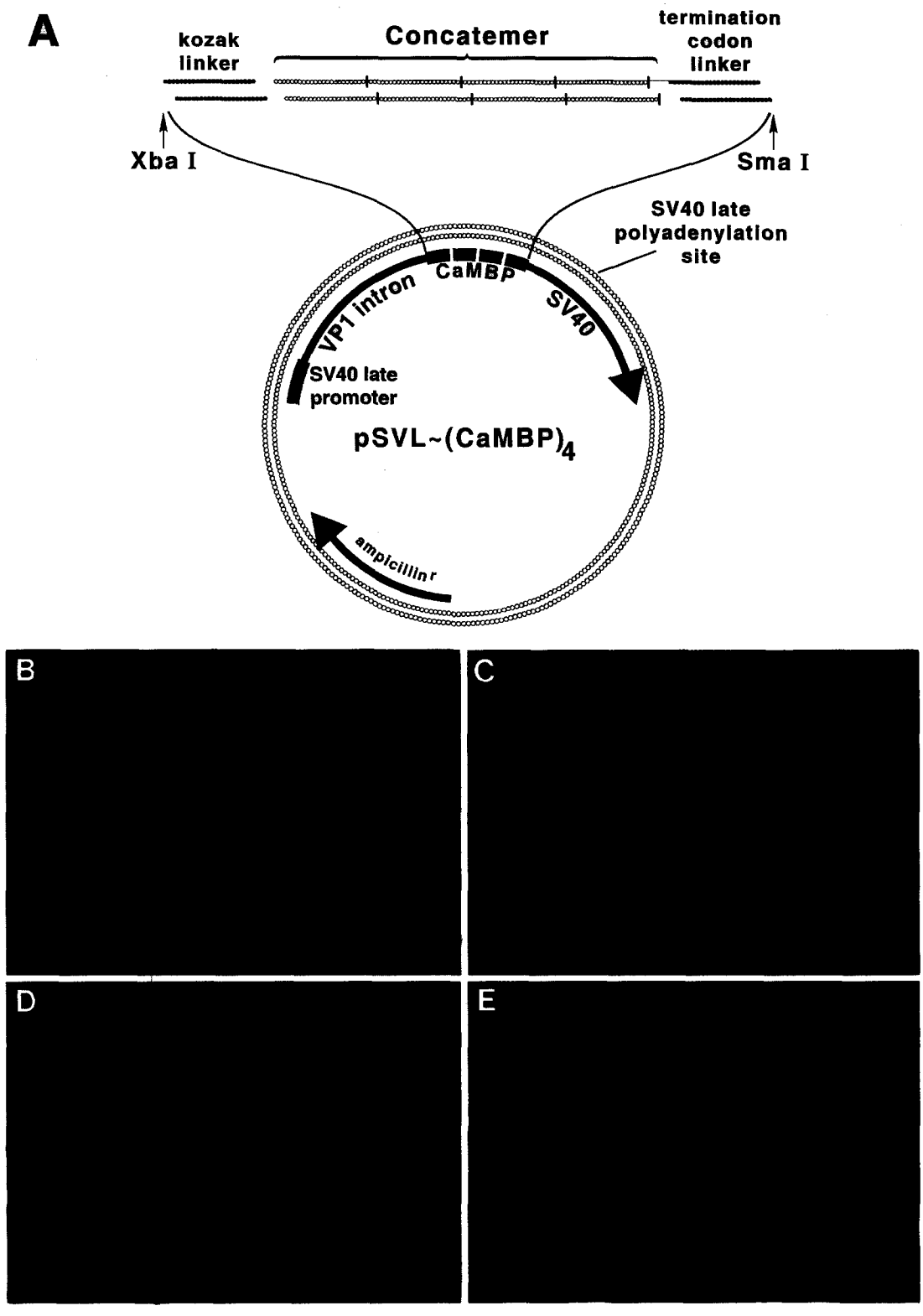

Fig. 4. Analysis of BrdU incorporation in COS-7 cells transfected with recombinant $(CaMBP)_4$ gene. (A) Construction of mammalian expression vector pSVL-$(CaMBP)_4$. A *Nhe*I-*Sma*I fragment (0.34 kb) containing the four repeat of CaM inhibitor peptide genes was ligated with 4.9 kb linearized *Xba*I-*Sma*I fragment of the mammalian expression vector pSVL. The expression construct was transiently transfected into exponentially growing COS-7 cells using the calcium precipitation method. Transfected COS-7 cells were incubated with BrdU for 1 h just before harvesting at 48 h after transfection. Cells were double immunostained with affinity-purified anti-CaM binding peptide antibody, followed by CY3-conjugated goat anti-rabbit IgG (B) and monoclonal antibody against BrdU, visualized by fluorescein-conjugated sheep anti-mouse IgG (C). (B) and (C) are the same field (20 ×). (D) Double exposure of double-immunostained transfected cells at high magnification (60 ×). (E) COS-7 cells transfected with parental plasmid pSVL. BrdU incorporation was detected with the same method as (C) (20 ×).

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