Biomedical Research 16 (1) 63-68, 1995

Inhibition of protein serine/threonine phosphatases directly induces premature chromosome condensation in mammalian somatic cells

EISUKE GOTOH[1,2], YOHKO ASAKAWA[1] and HIROSHI KOSAKA[3]

[1]Division of Genetic Resources, National Institute of Health Japan, Toyama, Shinjukuku, Tokyo 162, [2]Department of Radiology, The Jikei University School of Medicine, Nishishinbashi, Minatoku, Tokyo 105, and [3]Laboratory of Cell Growth and Differentiation, Tsukuba Life Science Centre, The Institute of Physical and Chemical Research (RIKEN), Koyadai, Tsukuba, Ibaraki 305, Japan

ABSTRACT

Chromosomes usually condense during mitosis, but condensation may be uncoupled from mitotic events under certain circumstances. This phenomenon is known as premature chromosome condensation (PCC). Here we show that 100 nM okadaic acid or calyculin A can induce PCC at any time during the cell cycle of several types of mammalian cells; G1-, S- and G2-PCC were observed. Both okadaic acid and calyculin A are specific inhibitors of type 1 and type 2A protein serine/threonine phosphatases, suggesting that these phosphatases play a central role in regulating chromosome condensation.

Chromosomes usually condense during mitosis, but when an interphase cell is fused in the mitotic cell, the interphase chromosomes condense prematurely (14). A similar premature chromosome condensation (PCC) can be induced by caffeine in cells blocked in S-phase (23). More recently, okadaic acid has also been reported to induce PCC in cultured cells blocked at S-phase (11, 24, 26) and in rapidly dividing mouse oocytes (9). A temperature-sensitive mutant of BHK21, (i.e. tsBN2), also undergoes PCC at the non-permissive temperature (17, 18).

However, it has not previously been reported that okadaic acid directly induces PCC in somatic cells without restrictive conditions such as blocking the cells at S-phase. Although the precise mechanism of PCC is not yet clear, the ability to induce it in normal somatic cells without any special pre-treatment may lead to the elucidation of the mechanisms of both the premature and the normal chromosome condensation in mitosis. Here we report that PCC can be induced by okadaic acid or calyculin A in mammalian cells at any stage of the cell cycle.

Abbreviations: PCC, premature chromosome condensation; BrdU, bromo-deoxyuridine; IL-2, interleukin-2; IL-3, interleukin-3; DMEM, Dulbecco's modified Eagle medium; MEM, minimal essential medium; Con A, concanavalin A; LPS, lipopolysaccharide; PHA-P, phytohemaggulutinin-P; PP1, type 1 protein phosphatase; PP2A, type 2A protein phosphatase; MPF, maturation promoting factor

Reagents

Okadaic acid and calyculin A were obtained from Wako Chemicals (Japan). Stock solutions of each chemical (1 mM in ethanol) were stored at −20°C. Bromo-deoxyuridine (BrdU) was obtained from Sigma (U.S.A.), as a 10 mM stock solution stored at 4°C. Phytohemaggulutinin-P (PHA-P), concanavalin A (Con A) and lipopolysaccharides (LPS) were obtained from Difco (U.S.A.), Sigma (U.S.A.) and Difco (U.S.A.), respectively.

Cell Culture and Chromosome Preparation

Peripheral blood was obtained by venipuncture from healthy donors. Lymphocytes were separated from whole blood using LymphoPrep (Becton Dickinson) according to the supplier's protocol. Separated peripheral blood lymphocytes were suspended in RPMI 1640 medium supplemented

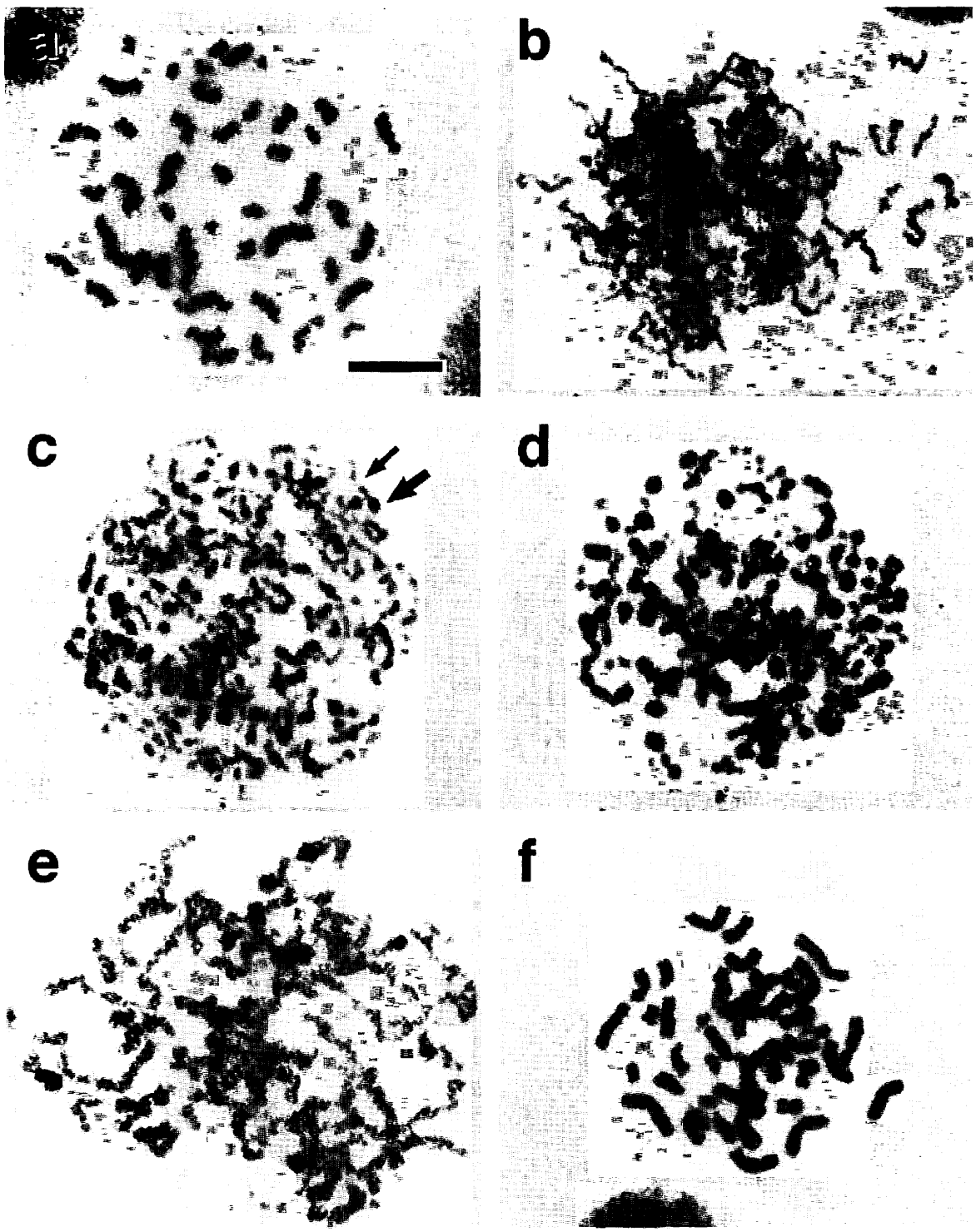

Fig. 1 PCC induced in human peripheral blood lymphocytes stimulated with phytohemaggulutinin-P by treatment with 100 nM okadaic acid for 2 h. a: Early G1-phase PCC. A well condensed metaphase-like shape but a univalent chromosome. b: Late G1-phase PCC. Chromatin is highly relaxed, but contains almost no thick section of chromosome (corresponding to replicating chromatin). c: S-phase PCC. Thick and thin portions of chromosomes are indicated by large and small arrows, respectively. d: A typical 'pulverised' form of S-phase PCC. e: G2-phase PCC. Bivalent chromosomes but not so condensed. f: G2-phase PCC. Chromosomes are well condensed and have a similar shape to metaphase chromosomes. Bar indicates 10 μm.

with 20% fetal calf serum (FCS) and then stimulated with PHA-P plus human interleukin-2 (IL-2) for 48 h. Human cell lines AT(L)5KY and AT(L)6KY were cultured exponentially in alpha-minimum essential medium supplemented with 10% FCS. All human cell cultures were conditioned in 5% CO_2 at 37°C. A single cell suspension from mouse spleens was obtained from 6-week-old C57bl/6 strain (13) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine and 5% (v/v) activated EL-4 supernatant (mouse IL-2) for 48 h. T and B lymphocytes were stimulated with 2.5 μg/ml Con A or 50 μg/ml LPS, respectively. The mouse cell line Ba/F3 was grown exponentially in DMEM supplemented with 10% FCS and 100 U/ml mouse recombinant interleukin-3 (IL-3; viability depends on IL-3). All mouse cell cultures were grown in 10% CO_2 at 37°C.

Cells were incubated for 2 h in 100 nM okadaic acid or calyculin A. As a simultaneous control, cells were grown in colcemid (0.1 μg/ml) instead of okadaic acid or calyculin A for 2 h. Cells were then harvested, resuspended in 75 mM KCl for 20 min, fixed with methanol: Acetic acid (3:1 vol/vol) 3 times, the cell suspension dropped on a glass slide and stained with a 5% Giemsa solution in 0.15 M phosphate-buffered saline (PBS) before image capture with a CCD camera attached to a Nikon microscope and processing using Adobe Photoshop on a Macintosh Quadra 950 computer.

BrdU Labelling and Diaminobenzidine Staining

Human peripheral blood lymphocytes were cultured as described above. Two and a half hours before harvesting, cells were pulse-labelled with 10 μM BrdU (final concentration) for 30 min. Cells were then washed 3 times with culture medium to remove free BrdU, 100 nM of okadaic acid added, and incubated for a further 2 h before fixation and staining as described above. Incorporated BrdU was detected as follows: Slides were immersed in 2 N HCl at room temperature for 30 min to denature DNA, and then immersed in 0.1 M $Na_2B_4O_7$, pH 8.5, for a few minutes to neutralise the acid. Slides were briefly washed twice in 0.5% Tween 20/PBS. Twenty microlitres of mouse anti-BrdU monoclonal antibody (Becton Dickinson), diluted with 50 μl 0.5% Tween 20/PBS, was added to the glass slide, and incubated at room temperature for 30 min in a humidified chamber. Slides were then washed twice with PBS. An aliquot of peroxidase-conjugated anti-mouse antisera (CAPPEL; 1:200 dilution in 0.5% Tween 20/PBS) was dropped onto each slide, and slides were incubated for further 30 min. Slides were washed 3 times with PBS and then subjected to diaminobenzidine reaction. Finally, slides were counterstained with 5% Giemsa solution in 0.15 M PBS and images were transmitted to a computer as described above.

Whilst studying primary cultures of human peripheral blood lymphocytes, we found that okadaic acid could induce PCC directly without blocking them in S-phase. Seventy-two hours after stimulating lymphocytes grown with PHA, the culture contains cells at various stages of the cell cycle. Fig. 1 shows the several kinds of PCC obtained after 2 h treatment with 100 nM okadaic acid. G1- and G2-PCC could be distinguished by their univalent or bivalent chromosomes (Fig. 1, a, b, e and f). S-phase PCC had a characteristic mixture of thick and thin chromosomal parts, corresponding to bivalent and univalent replicated chromatin (Fig. 1c) and a 'pulverised' appearance (Fig. 1d), as described elsewhere (21).

The presence of replication chromatin in the S-PCC was confirmed by labelling with BrdU and staining using the DAB reaction; incorporated BrdU in the S-phase PCC appears brown (Fig. 2); 25.0% (8/32) of cells were induced to undergo PCC and 15.6% (5/32) showed S-phase PCC (Fig. 2).

We next considered whether another inhibitors of both PP1 and PP2A could induce PCC at any phase of the cell cycle and whether the effect was cell type-specific. Accordingly, a similar series of experiments was performed on human peripheral blood lymphocytes, two established human cell lines, i.e. AT(L)5KY and AT(L)6KY, mouse spleen cells stimulated with Con A or LPS, and the mouse cell line Ba/F3 using 100 nM okadaic acid or calyculin A.

Although there were differences in frequency of PCC, all cell types were susceptible to okadaic acid, although mouse cells were more resistant than human cells (Fig. 3). However, mouse cell lines responded strongly to calyculin A. Calyculin A (100 nM) was cytotoxic to most mouse spleen cells but almost all surviving cells underwent PCC (> 90%; data not shown). Calyculin A at 10 nM exerted less cytotoxicity and induced considerable PCC.

The frequency of PCC obtained with either okadaic acid or calyculin A was higher in all cell lines examined (ranging from 11.5% to 99.2%) than the frequency of metaphase obtained after treating the

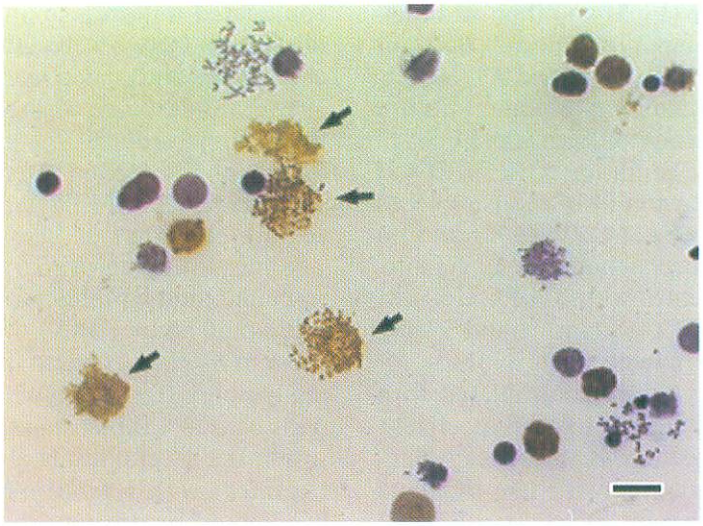

Fig. 2 Various phases of S-PCC after incorporation of BrdU, and staining sites containing analogs. The S-PCC (arrows) appears light to dark brown in colour, depending on the degree of incorporation of BrdU. Bar indicates 20 μm.

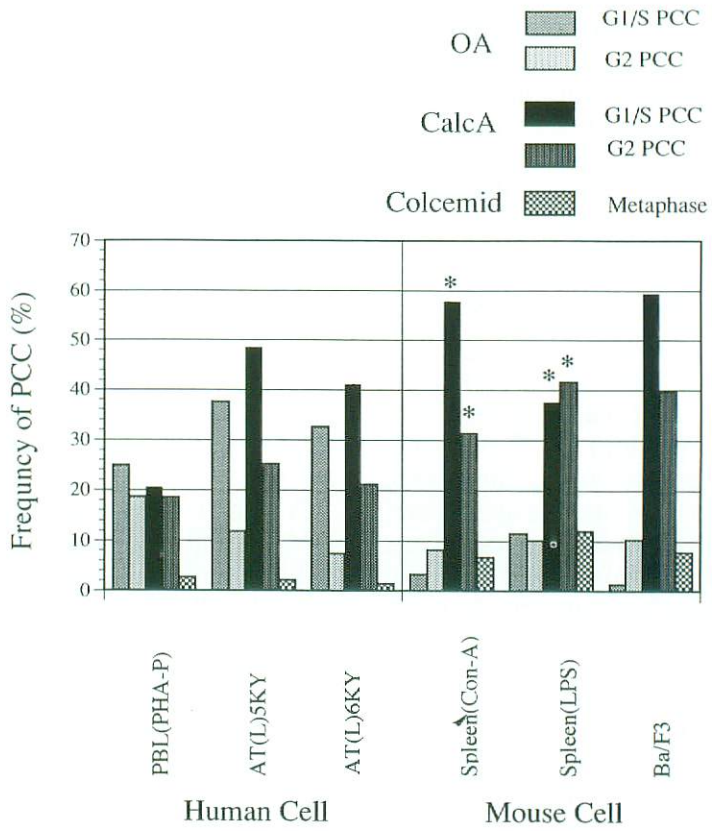

Fig. 3 Frequencies of G1-, S- and G2-PCC obtained after treatment of various cells with 100 or 10* nM okadaic acid or calyculin A

same cultures with colcemid (1.4% to 12.0%; control study); these inhibitors effectively condense chromosomes. The different responses of human and mouse cells are probably attributable to differences in the sensitivity, permeability, or other factors.

In this study, we have shown that okadaic acid and calyculin A directly induce PCC in several types of mammalian somatic cells. Okadaic acid and calyculin A are specific inhibitors of type 1 and type 2A protein serine/threonine phosphatases (PP1, PP2A) (2-4). So PCC is probably induced by the inhibition of PP1 and/or PP2A. The phenomenon of PCC was first demonstrated by cell fusion with cells arrested in mitosis, suggesting that some factor (s) accumulates in mitotic cells and drives PCC. Several other findings imply protein phosphatases in chromosome condensation.

Germinal vesicle breakdown (GVBD) is a phenomenon analogous to PCC that occurs during the maturation of the oocytes. Here maturation promoting factor (MPF), a $P34^{cdc2}$/cyclin complex with kinase activity, is believed to drive GVBD because the microinjection of anti-$P34^{cdc2}$ antibodies blocks the process (22).

Treatment of *Xenopus* oocyte extract with okadaic acid activates MPF (10), and microinjection of okadaic acid induces the maturation of oocytes (12). Furthermore, mutation of PP1 or PP2A produced abnormal mitotic phenotypes including chromosome condensation (1, 7, 8, 15, 19, 20). The precise function of protein phosphatases, however, remains unclear.

Our results have practical implications. Several findings of radiation-induced cleavage of chromosomes have been reported using the PCC technique (5, 6, 16, 25). Current methods of PCC induction require a laborious cell fusion, involving synchronising and collecting large amounts of mitotic cells followed by fusion with target cells. It is often difficult to obtain metaphase chromosomes using colcemid which restricts analysis. This facile method of inducing PCC allow the analysis of the dynamics of replication fork movement in S-phase (Asakawa and Gotoh, Cold Spring Harbor Laboratory Meeting, The Cell Cycle 1994; manuscript in preparation) and breakage induced by irradiation during interphase.

We thank Yukio Nakamura for the gift of the Ba/F3 cell line. We are also grateful to Peter R. Cook, Antony T. Orton and Elizabeth Carr for critical reading and correcting of our manuscript. AT(L)5KY and AT(L)6KY cell lines were donated by the Japanese Cancer Research Resources Bank (JCRB). Y. A. is supported by a Research Resident Fellowship from the Foundation for the Promotion of Cancer Research.

Received 14 November 1994; and accepted 28 November 1994

REFERENCES

1. Axton J. M., Dombradi V., Cohen P. T. W. and Glover D. M. (1990) One of the protein phosphatase 1 isoenzymes in *Drosophila* is essential for mitosis. *Cell* **63**, 33-46
2. Bialojan C. and Takai A. (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* **256**, 283-290
3. Cohen P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**, 453-508
4. Cohen P., Holmes C. F. B. and Tsukitani Y. (1990) Okadaic acid: A new probe for the study of cellular regulation. *TIBS* **15**, 98-102
5. Cornforth M. N. and Bedford J. S. (1983) X-ray-induced breakage and rejoining of human interphase chromosomes. *Science* **222**, 1141-1143
6. Cornforth M. N. and Bedford J. S. (1985) On the nature of a defect in cells from individuals with ataxia-telangiectasia. *Science* **227**, 1589-1591
7. Dombradi V., Axton J. M., Barker H. M. and Cohen P. T. W. (1990) Protein phosphatase 1 activity in *Drosophila* mutants with abnormalities in mitosis and chromosome condensation. *FEBS Lett.* **275**, 39-43
8. Doonan J. H. and Morris N. R. (1989) The bimG gene of *Aspergillus nidulans*, required for completion of anaphase, encodes a homolog of mammalian phosphoprotein phosphatase 1. *Cell* **57**, 987-996
9. Dyban A. P., De-Sutter P. and Verlinsky Y. (1993) Okadaic acid induces premature chromosome condensation reflecting the cell cycle progression in one-cell stage mouse embryos. *Mol. Reprod. Dev.* **34**, 402-415
10. Felix M., Cohen P. and Karsenti E. (1990) Cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cell cycle: Evidence from the effects of okadaic acid. *EMBO J.* **9**, 675-683
11. Ghosh S., Paweletz N. and Schroeter D. (1992) Failure of kinetochore development and mitotic spindle formation in okadaic acid-induced premature mitosis in HeLa cells. *Exp. Cell Res.* **201**, 535-540
12. Goris J., Hermann J., Hendrix P., Ozon R. and Merlevede W. (1989) Okadaic acid, a specific protein phosphatase inhibitor, induces maturation and MPF formation in *Xenopus laevis* oocytes. *FEBS Lett.* **245**, 91-94
13. Hunt S. V. (1987) Preparation of lymphocytes and accessory cells. In *Lymphocytes: A Practical Approach* (ed. Klaus G. G. B.) IRL Press Limited, Oxford, pp. 1-34
14. Johnson R. T. and Rao P. N. (1970) Mammalian cell fusion: Induction of premature chromosome condensation in interphase nuclei. *Nature* **226**, 717-722
15. Kinoshita N., Ohkura H. and Yanagida M. (1990) Distinct, essential roles of type 1 and 2A protein phosphatases in the control of the fission yeast cell division cycle. *Cell* **63**, 405-415
16. Kovacs M. S., Evans J. W., Johnstone I. M. and Brown J.

M. (1994) Radiation-induced damage, repair and exchange formation in different chromosomes of human fibroblasts determined by fluorescence in situ hybridization. *Radiat. Res.* **137**, 34-43
17. NISHIMOTO T. and BASILICO C. (1978) Analysis of a method for selecting temperature sensitive mutants of BHK cells. *Somat. Cell Genet.* **4**, 323-339
18. NISHIMOTO T., EILEN E. and BASILICO C. (1978) Premature chromosome condensation in a ts DNA$^-$ mutant of BHK cells. *Cell* **15**, 475-483
19. OHKURA H., KINOSHITA N., MIYATANI S., TODA T. and YANAGIDA M. (1989) The fission yeast dis2+ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. *Cell* **57**, 997-1007
20. OHKURA H. and YANAGIDA M. (1991) S. pombe gene sds22+ essential for a midmitotic transition encodes a leucine-rich repeat protein that positively modulates protein phosphatase-1. *Cell* **64**, 149-157
21. RAO P. N., WILSON B. and PUCK T. T. (1977) Premature chromosome condensation and cell cycle analysis. *J. Cell Physiol.* **91**, 131-142
22. RIABOWOL K., DRAETTA G., BRIZUELA L., VANDRE D. and BEACH D. (1989) The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell* **57**, 393-401
23. SCHLEGEL R. and PARDEE A. B. (1986) Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. *Science* **232**, 1264-1266
24. STEINMANN K. E., BELINSKY G. S., LEE D. and SCHLEGEL R. (1991) Chemically induced premature mitosis: Differential response in rodent and human cells and the relationship to cyclin B synthesis and p34cdc2/cyclin B complex formation. *Proc. Natl. Acad. Sci. USA* **88**, 6843-6847
25. VYAS R. C., DARROUDI F. and NATARAJAN A. T. (1991) Radiation-induced chromosomal breakage and rejoining in interphase-metaphase chromosomes of human lymphocytes. *Mutat. Res.* **249**, 29-35
26. YAMASHITA K., YASUDA H., PINES J., YASUMOTO K., NISHITANI H., OHTSUBO M., HUNTER T., SUGIMURA T. and NISHIMOTO T. (1990) Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. *EMBO J.* **9**, 4331-4338