

Chromosome Condensation Outside of Mitosis: Mechanisms and New Tools

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A basic principle of cell physiology is that chromosomes condense during mitosis. However, condensation can be uncoupled from mitotic events under certain circumstances. This phenomenon is known as “premature chromosome condensation (PCC).” PCC provides insights in the mechanisms of chromosome condensation, thus helping clarifying the key molecular events leading to the mitosis. Besides, PCC has proved to be an useful tool for analyzing chromosomes in interphase. For example, using PCC we can visualize genetic damage shortly after the exposure to clastogenic agents. More than 30 years ago, the first report of PCC in interphase cells fused to mitotic cells using Sendai virus was described (virus-mediated PCC). The method paved the way to a great number of fundamental discoveries in cytogenetics, radiation biology, and related fields, but it has been hampered by technical difficulties. The novel drug-induced PCC method was introduced about 10 years ago. While fusion-induced PCC exploits the action of external maturation/mitosis promoting factor (MPF), migrating from the inducer mitotic cell to the interphase recipient, drug-induced PCC exploits protein phosphatase inhibitors, which can activate endogenous intracellular MPF. This method is much simpler than fusion-induced PCC, and has already proven useful in different fields. *J. Cell. Physiol.* 209: 297–304, 2006. © 2006 Wiley-Liss, Inc.

CHROMOSOME CONDENSATION IN INTERPHASE

In ordinary circumstances, chromosomes condense in mitotic phase only, under highly ordered molecular events. However, in certain circumstances, chromosome condensation may be uncoupled from mitotic events. This phenomenon is known as premature chromosome condensation (PCC). The first observation of PCC was reported in virus mediated multinucleated fused cells of interphase and mitotic cells (Kato and Sandberg, 1967). They observed a typical appearance of “shattered” or “pulverized” chromosomes following virus infection of cells, and they thought that the “pulverized” chromatin represented chromosomal fragments induced by the virus. The first correct interpretation of this phenomenon was given by Johnson and Rao following their carefully designed experiment (Johnson and Rao, 1970). They fused synchronized interphase cells (in G1, S, or G2 phase) to mitotic cells using Sendai virus (Fig. 1), and they found that the appearance of condensed chromatin was dependent on the cell phase of interphase cells at that time of fusion; that is, (1) G1-phase nuclei produced univalent chromosomes, (2) G2-phase nuclei showed bivalent chromosomes, similar to mitotic chromosomes, although less condensed, and (3) S-phase nuclei converted to a “pulverized” appearance that consisted of univalent and bivalent chromosomes. They concluded that the interphase chromatin was condensed “prematurely” following fusion to mitotic-like chromosomes, and they first named this phenomenon as “premature chromosome condensation (PCC),” and the condensed interphase chromatins “prematurely condensed chromosomes” (PCCs) (Fig. 2). PCC was soon recognized as a valuable tool for investigations in chromosome dynamics, such as in radiation cytogenetics and chromosome replication studies (Hittelman and Rao, 1974; Schor et al., 1975; Hittelman and Pollard, 1982; Mullinger and Johnson, 1983; Cornforth and Bedford, 1983a). Virus-induced fusion PCC method has, however,

several drawbacks. First, fusion efficiency is highly dependent on the virus strain and viral activity. Second, the technique cannot be always applied, because the cell should express surface receptors to the fusogenic virus. Third, the chromosomes are somewhat confusing mixture of mitotic inducer and recipient cells, and differential replication staining (in mitotic cells) is needed to visualize the genetic material in the two cells (Cornforth and Bedford, 1983b). Finally, handling of viruses restricts the use of virus-mediated PCC method, and manipulation of the infectious virus is generally banned in non-specialized laboratories. Cell fusion can also be achieved by chemicals (Fig. 2), in particular polyethylene glycol (PEG), although the fusion yield is generally lower than that obtained by virus. Pantelias and Maillie (1983) first successfully applied PEG-fusion to PCC induction, and this method has been widely used in several laboratories, especially for radiation cytogenetics studies (Pantelias and Maillie, 1984; Iliakis et al., 1987; Suzuki et al., 1992; Watanabe et al., 1992; Okayasu and Iliakis, 1993; Durante et al., 1996b, 1998b).

DRUG-INDUCED PCC

As described in the previous section, PEG- or virus-induced PCC (cell fusion-mediated PCC) is a useful method for chromosome analysis, but its use is problematic. Due to these restrictions, virus or PEG-mediated fusion-PCC is used in very limited number of laboratories only.

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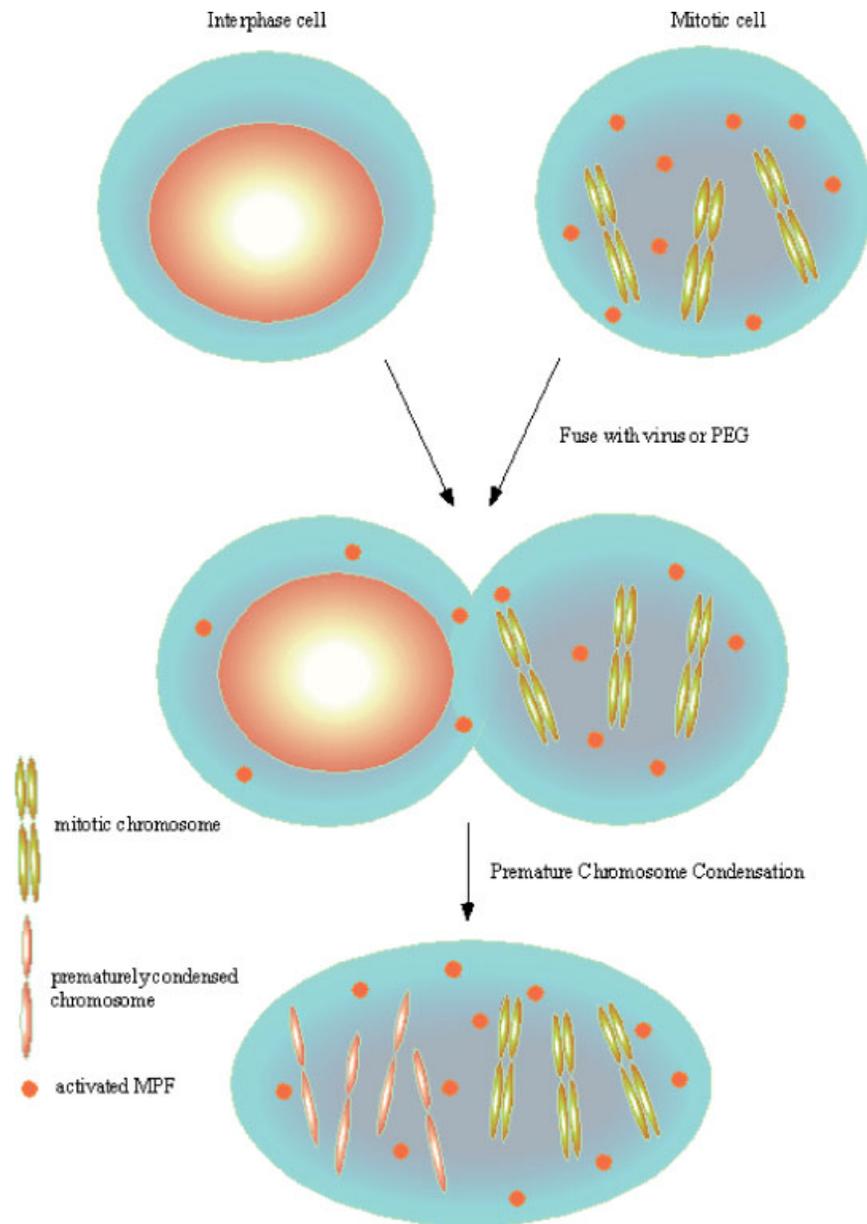


Fig. 1. Mechanism of fusion-induced PCC. Fusion is generally achieved either by virus or chemicals (PEG).

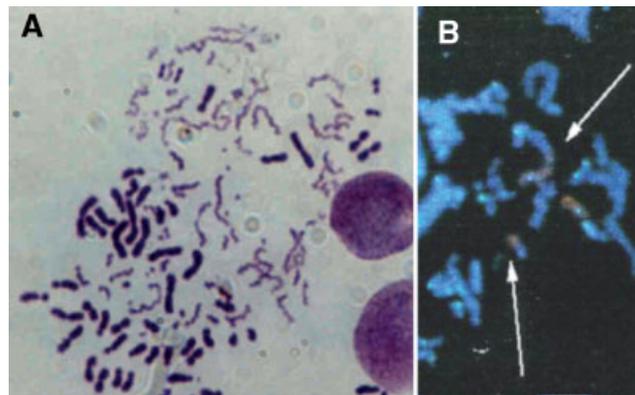


Fig. 2. Photomicrographs of fusion-induced PCCs. The images show prematurely condensed single-chromatid (i.e., univalent) human chromosomes from G0 peripheral blood lymphocytes fused by PEG, after exposure to 7 Gy γ -rays, to mitotic Chinese hamster cells. Note that the chromosomes are mixture of mitotic hamster cell and recipient human cell. **A:** Several fragments are visible by Giemsa-staining in the irradiated human chromosomes (pale purple).

B: A dicentric (with associated acentric fragment, both pointed by arrows) in human PCC 4, visualized by FISH-painting with a human DNA whole chromosome probe (spectrum orange) combined to a pan-centromeric probe (spectrum green). Photographs from References Durante et al. (1996a,b), copyright Radiation Research Society, reproduced with permission.

Several approaches have been carried out to induce PCC using chemicals, which would make the procedure easier and reliable. First successes were obtained with caffeine and okadaic acid, but cells had to be synchronized in S-phase using DNA synthesis inhibitors such as hydroxyurea or thymidine (Schlegel and Pardee, 1986; Schlegel et al., 1990; Yamashita et al., 1990). The obtained chromosomes were, therefore, S-phase PCC only, which cannot be used for usual chromosome analysis, because they appear pulverized. The first report of PCC induction in somatic cells in any phase of cell cycle, without arresting the cells in S-phase, came from Gotoh et al. (1995). The authors used calyculin A or okadaic acid, specific inhibitors of type 1 and 2A protein phosphatases (Bialojan and Takai, 1988; Ishihara et al., 1989; Cohen et al., 1990) (Fig. 3). Calyculin A is, in particular, able to induce PCC in many kinds of cells, either in suspension or attached, and the PCC index is generally higher than with other protein phosphatase inhibitors. The method of drug-induced PCC is relatively easy whereby simply substitutes the use of colcemid to calyculin A in conventional chromosome preparation protocol (Gotoh et al., 1995; Durante et al., 1998a). Incubation time of only approximately 30 min, much shorter than colcemid block (2–4 h) can induce a substantial number of PCCs. In addition, PCC index (corresponds to mitotic index) is usually much higher (>20%) than mitotic index of colcemid protocol (~1–2%) (Gotoh et al., 1995; Asakawa and Gotoh, 1997; Durante et al., 1998a). Examples of PCCs in different phases of the cell cycle, visualized by Giemsa-stained or fluorescence in situ hybridization (FISH) with whole-chromosome human DNA probes, are given in Figure 4. Drug-induced PCC is becoming increasingly popular, and a lot of reports in the literature using this technique have been recently published, where this technique is applied

to several mammalian cells, either attached or in suspension (Gotoh and Asakawa, 1996; Asakawa and Gotoh, 1997; Coco-Martin and Begg, 1997; Durante et al., 1998a, 1999; Gotoh et al., 1999, 2005; Prasanna et al., 2000; Ito et al., 2002; Bezrookove et al., 2003; Terzoudi et al., 2003; Malik et al., 2004; Blakely et al., 2005; El Achkar et al., 2005; Someya et al., 2006).

MOLECULAR MECHANISMS OF CHROMOSOME CONDENSATION

The mechanism of chromosome condensation has been deeply investigated and several key molecules, such as condensin, cohesin, lamina, microtubules, histone H1, aurora kinase, and topoisomerase II (see for instance Kubiak, 1991; Rattner and Wang, 1992; Ishida et al., 1994; Van Hooser et al., 1998; Vass et al., 2003; Maton et al., 2005), have been identified and their mechanism described. However, the complete mechanism is still unclear. PCC is partly similar, yet not quite the same phenomenon as normal chromosome condensation (Ghosh et al., 1992; Rattner and Wang, 1992; Guo et al., 1995). It is expected that a deeper knowledge of PCC may lead to a better understanding of the normal mitotic chromosome condensation.

Incunabula, it was thought that PCC (chromosome pulverization) was caused by the nature of viruses used for cell fusion (Kato and Sandberg, 1967), because at that time pulverization of chromosome was thought to be a quintessence matter. However, as described above, this idea was turned over by the carefully designed experiment using synchronized cells (Johnson and Rao, 1970). They interpreted the mechanism of PCC as follows: (1) "certain molecules," which promote chromosome condensation, accumulate in mitotic nuclei, (2) these molecules work in the interphase cell nuclei after

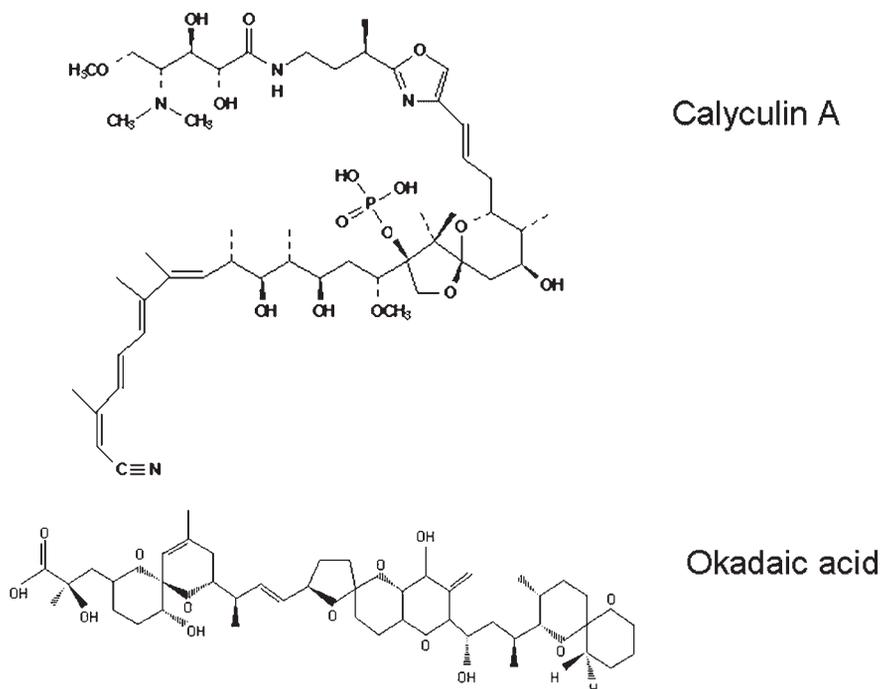


Fig. 3. Molecular structures of calyculin A and okadaic acid, two powerful protein phosphatase inhibitors and PCC inducers. Chemical characteristics are: Calyculin A: Molecular Formula, $C_{50}H_{81}N_4O_{15}P$; Molecular Weight, 1009.18; IC_{50} for PP1, 0.5–2 nM; IC_{50} for PP2A, 0.1–1 nM; Source, *Discodermia Calyx*. Okadaic Acid: Molecular Formula, $C_{44}H_{68}O_{13}$; Molecular Weight, 805.2; IC_{50} for PP1, 10–60 nM; IC_{50} for PP2A, 0.1–1 nM; Source, *Halichondria Okadai*.

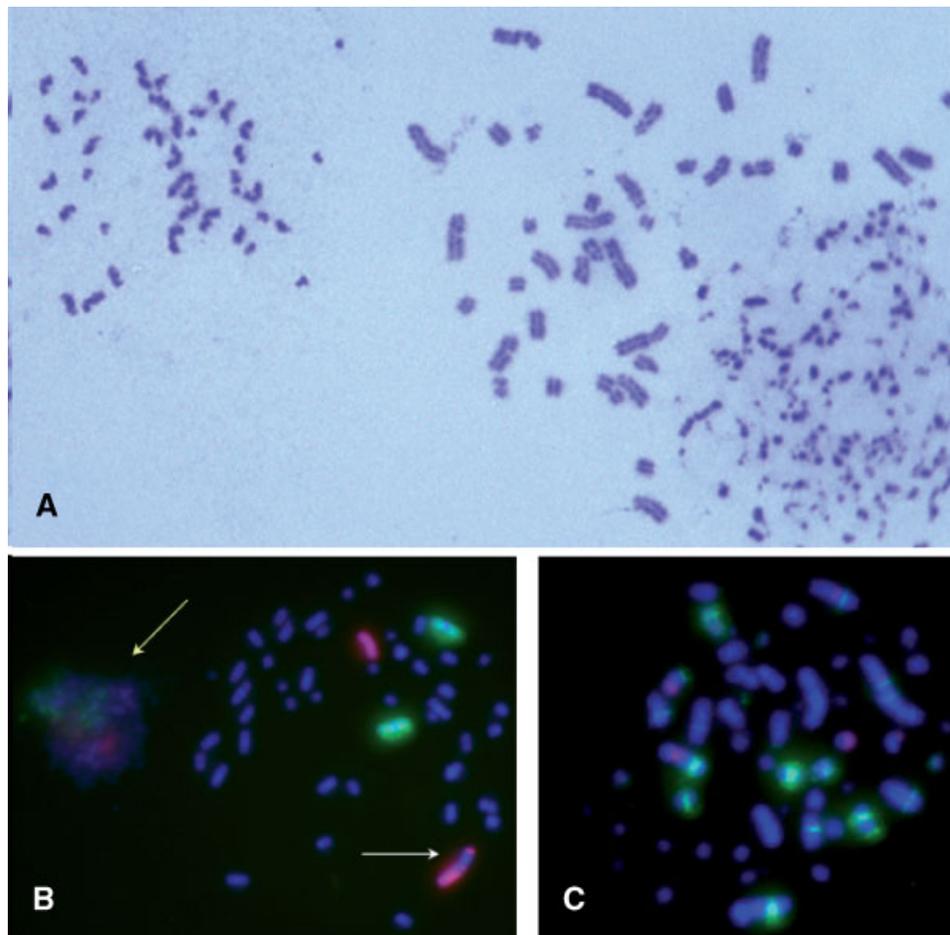


Fig. 4. Examples of PCC in human mononuclear lymphocytes induced by calyculin A. **A:** A Giemsa-stained image of three cells in the same area of the slide in three different phases of the cell cycle: left, univalent G1 chromosomes; center, bivalent G2 chromosomes; right, pulverized S-phase cell. **B:** An insertion in chromosome 2, FISH-painted in spectrum orange (pointed by a white arrow) in a cell from a patient during radiotherapy. The normal chromosome 1 is painted in

green. An S-phase cell is also visible in the same image, pointed by the yellow arrow. Drug-induced PCC is preferable to conventional colcemid-block for biodosimetry after partial-body exposure, such as in radiotherapy. **C:** A multi-aberrant cell, showing several complex rearrangements in both chromosomes 1 and 2, followed exposure to 1 Gy of high-energy iron ions. Severe damage induced by densely ionizing radiation may be underestimated using metaphase analysis.

fusion to metaphase cells (3) the migrating molecules force the interphase chromosomes to condense prematurely (Johnson and Rao, 1970). The premature condensation process takes less than 1 h to complete: approximately 20 min are necessary for the condensation to proceed to a point where no DNA rejoining is possible (Cornforth and Bedford, 1993; Durante et al., 1998c).

The germinal vesicle breakdown (GVBD) is a similar phenomenon, where oocyte chromosomes condense prematurely at meiotic metaphase stage (egg maturation). The maturation-promoting factor (MPF) was first identified as the key molecule inducing GVBD induction (Masui, 1974, 1982, 2001). It was later revealed that MPF regulates chromosome condensation not only in meiotic but also in mitotic cells (Miake-Lye and Kirschner, 1985), therefore, MPF was re-named as maturation/mitosis promoting factor. MPF is actually a complex of p34cdc2/cyclinB (Dunphy et al., 1988; Gautier et al., 1988; Maller et al., 1989) and plays a central role in cell cycle regulation and cell growth control. The dephosphorylated form of p34cdc2/cyclinB complex (activated MPF) promotes chromosome condensation (Moreno et al., 1989). Therefore, after cell fusion by either viruses or PEG, the interphase nucleus in the fused cell is exposed to activated MPF which is

supplied from the mitotic nucleus, thus resulting in prematurely chromosome condensation of interphase cell chromatin (Fig. 1).

Cdc25 is a cell cycle checkpoint protein which regulates the onset of mitosis along with p34cdc2/cyclinB (Ducommun et al., 1990; Moreno et al., 1990). Cdc25 is a tyrosine phosphatase that activates p34cdc2/cyclinB complex by dephosphorylation of tyrosine residue (Gould et al., 1990; Dunphy and Kumagai, 1991; Gautier et al., 1991; Kumagai and Dunphy, 1991; Strausfeld et al., 1991). The activity of cdc25 is regulated by auto-phosphorylation/dephosphorylation and is sensitive to PP1 and PP2A (type 1 and 2A protein phosphatase) (Izumi et al., 1992; Kumagai and Dunphy, 1992; Kinoshita et al., 1993). Okadaic acid and calyculin A, specific inhibitors of PP1 and PP2A, influence the activity of cdc25 and p34cdc2/cyclinB (Fig. 5), thus promoting the premature entry in mitotic stage (Kumagai and Dunphy, 1992; Kinoshita et al., 1993). Activated MPF (p34cdc2/cyclinB) may finally promote chromosome condensation in interphase. In this hypothesis, PCC is not directly induced by the chemicals, but should be caused by inhibition of protein phosphatase activity. Interestingly, other inhibitors of type 1 and type 2A protein phosphatase such as endothal and cantharidine are also able to induce PCC (Gotoh, 1996, 1998).

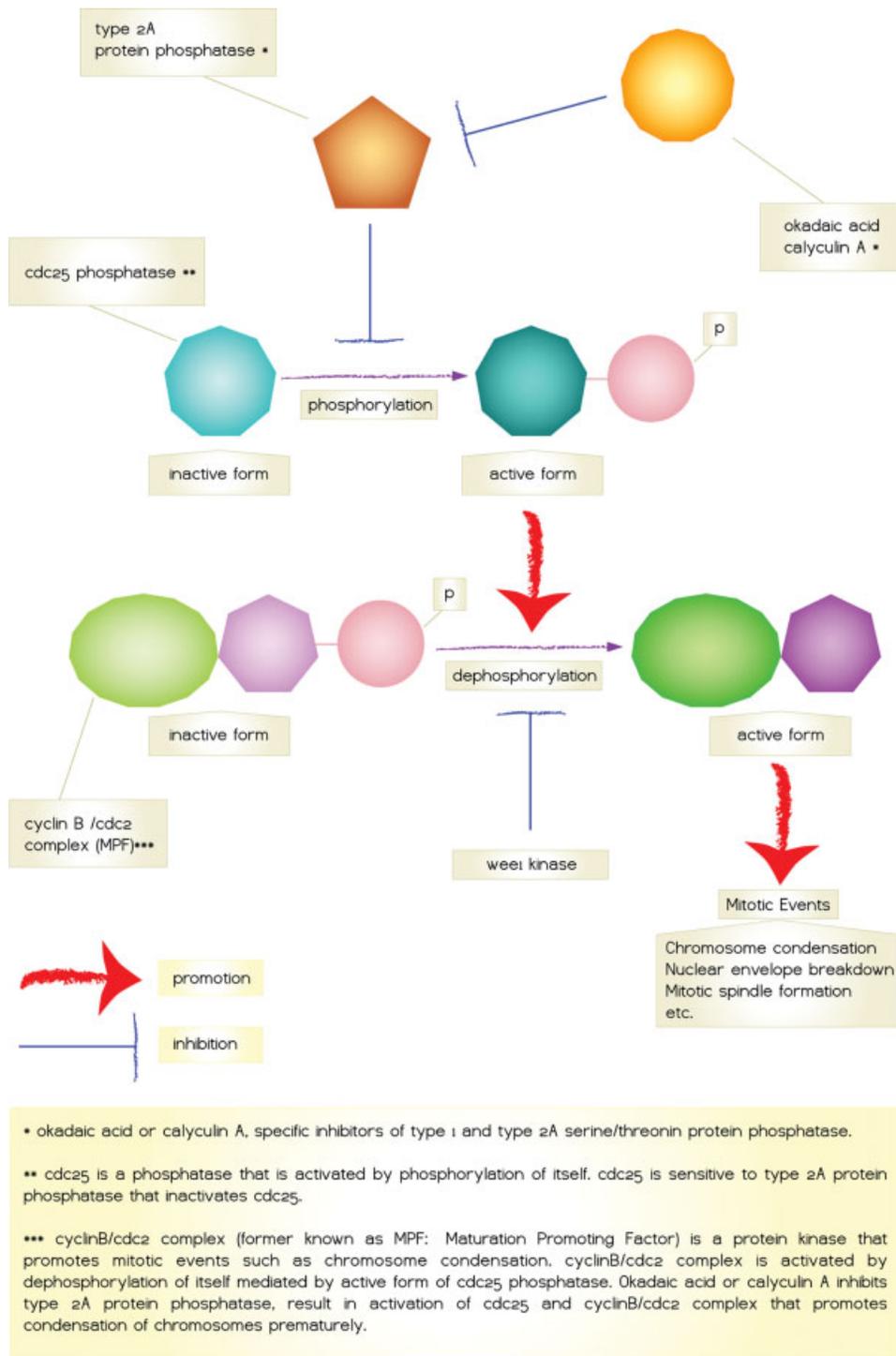


Fig. 5. Mechanism of chromosome condensation induced by protein phosphatase inhibitors.

Clearly, according to the mechanism described above, PCC will only be induced if MPF protein is available in the cell. It is well known that MPF activity is dependent on the cellular concentration of cyclins, in particular cyclin B (e.g., Doree and Galas, 1994). Cyclin B concentration oscillates through cell cycle: it is very low in G1, and increases gradually from S to G2, where it reaches its maximum, corresponding to the maximum in MPF activity (Fig. 6). Therefore, calyculin A or okadaic acid will induce PCC predominantly in G2-phase,

followed by S-phase and only few G1-phase. A number of late telophase post-mitotic G1 are still visible by drug-induced PCC, but no PCC can be induced in G0 cells (Durante et al., 1998a). PCC induced by calyculin A in G2 takes only about 5 min (Gotoh et al., 1999), that is, it is probably faster than fusion-induced PCC (about 20 min).

The lack of G0-phase PCC after calyculin A or okadaic acid can represent a drawback as compared to fusion-induced PCC. In fact, if early events in G0 cells want to

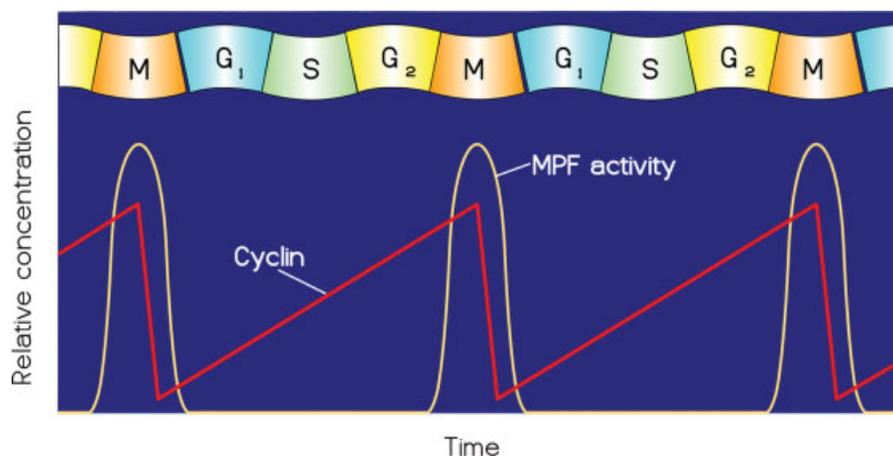


Fig. 6. Relationship between cyclin concentration and MPF activity during the cell cycle.

be studied, fusion-induced PCC is to be preferred to calyculin A. For instance, the kinetics of DNA repair in G0 cells exposed to ionizing radiation has been extensively studied using fusion-induced PCC (Cornforth and Bedford, 1983a, 1993; Durante et al., 1998b,c), while calyculin A was used to measure G2 kinetics (Gotoh et al., 1999; Kawata et al., 2000, 2004).

Efforts to induce PCC in G0, resting-state human cells have concentrated on other key molecules. Since cyclin B concentration is too low in G0, Prasanna et al. (2000) added adenosine triphosphate (ATP) and p34cdc2/cyclin B kinase to okadaic acid to induce PCC in G0 human lymphocytes. Although the authors observed condensation indexes as high as 40% at high concentrations of p34cdc2/cyclin B, the quality of the chromosome spread is hardly suitable for karyotyping or detection of structural chromosome aberrations. Nevertheless, the same authors have demonstrated that FISH-painting of these partly condensed spreads produce distinct color-domains for each painted chromosome pair, and radiation-induced breaks and rearrangements result in an increased number of hybridized spots (Prasanna and Blakely, 2005). This technique is then potentially very useful in biological dosimetry of ionizing radiation, especially when results are needed shortly after the exposure.

PCC APPLICATIONS

The conventional colcemid-block protocol has been used popularly for analyzing chromosomes. Cytological material is, however, limited to mitotic chromosomes. In fact, colcemid inhibits the assembly of spindle body at mitotic phase, thus the cells arrest in mitosis with condensed chromosomes. In contrast, PCC forces the chromosomes to be condensed “prematurely” not only in mitosis but also in interphase nuclei, hence interphase nuclei are visualized as condensed chromosome form. Because of this unique property, PCC has been used for analyze various chromatin events occur during interphase, including (1) breakage and repair of chromosomes following exposure to either ionizing radiation (Cornforth and Bedford, 1983a; Pantelias, 1986; Iliakis et al., 1987; Bedford and Goodhead, 1989; Durante et al., 1996a) or chemical mutagens (Hittelman and Rao, 1974, 1975; Hittelman and Pollard, 1982; Sognier and Hittelman, 1986); (2) DNA replication (Hanks and Rao, 1980; Lau and Arrighi, 1980, 1981; Mullinger and Johnson, 1983), (3) conformational changes during cell cycle

(Schor et al., 1975; Rao et al., 1977; Hittelman and Rao, 1978; Rao and Hanks, 1980; Hanks et al., 1983), or others. Drug-induced PCC has also been applied in several studies. PCC is extremely useful in biodosimetry of ionizing radiation, especially in a number of cases where metaphase analysis can fail. The application of PCC is proving the worth in overcoming some of the limitations of the conventional dicentric assay (IAEA, 2001). These include (Durante et al., 1997): partial body exposure, such as in radiotherapy, when unirradiated cells will be harvested sooner than exposed cells, delayed by radiation-induced cell-cycle blocks; exposure to densely ionizing radiation, which produces prolonged cell-cycle delays; very low mitotic indexes, such as in pathological situations, in the elderly, or in stress conditions; exposure to very high radiation dose, which invariably produce poor or no mitosis after stimulation.

A simple protocol for simultaneous analysis of interphase–metaphase chromosomes in human lymphocytes for biological dosimetry was described by Durante et al. (1998a), and since then has been widely applied to biodosimetry. In cancer patients undergoing radiotherapy, use of PCC induced by calyculin A resulted in accurate measurements of the yield of chromosomal aberrations in lymphocytes of patients during the treatment at different sites and with different radiation qualities (Durante et al., 2000). In all cases, the dose-response curve shows a saturation at high number of fractions, due to re-population from unirradiated bone marrow. In addition, it has been shown that the aberration yield strongly depends on the number of lymph nodes in the radiation field (d’Alesio et al., 2003), thus suggesting that most lymphocytes are not exposed while in the circulating blood, but in the lymph nodes.

For accidental overexposure to very high doses, dose estimates should be determined as quickly as possible to choose appropriate medical treatments for the victims. However, when human body is irradiated with large doses, the white cells remaining in the peripheral blood not only arrest at G2 or G1 phase and do not enter mitosis, but also undergo mitotic cell death or apoptosis. As a result, it is usually impossible to prepare chromosome samples for analysis. Before the 21st century, exposures over 10 Gy were invariably lethal (~2 times higher than LD50 of 6 and 4 Gy of humans with medical care or without medical care, respectively, Anno et al., 2003). Thus it was not practically required for the cytogenetic biodosimeter to be able to estimate whole

body doses over 10 Gy. However, with recent advances in medical technology such as stem cell transplantation and cytokine therapy, it became possible to save victims exposed to over 10 Gy whole body (Lloyd, 1997). Therefore, the need for developing a cytogenetic biodosimeter that covers the estimation doses over 10 Gy been emerged. This dose limitation of biodosimeter was first overcome using drug-induced PCC technique, where the maximum estimable dose is approximately 40 Gy γ -rays if combined to chromosome painting method (Gotoh and Asakawa, 1996). Following this report, other parameters have been proposed to analyze prematurely condensed chromosomes after very high doses: centric rings (Kanda et al., 1999), combined use of total chromosome number and G2-PCC index (Gotoh et al., 2005), and the length ratio of longest/shortest size of chromosome (Gotoh and Tanno, 2005). The drug-induced PCC has been indeed applied after the 1999 criticality accident at the uranium fuel conversion test facility of JCO in Tokai-mura, Japan. Three workers were exposed to very high doses (two of them died within a few months), and ^{24}Na and ^{32}P radioactivity measurements by whole-body counters suggested soon that two of them were exposed to lethal doses (Miyamoto et al., 2002). However, cytogenetic analysis by drug-induced PCC provided the best estimate of the absorbed dose (Kanda et al., 2002).

Further applications of drug-induced PCC include (1) mutagenic assays (Asakawa and Gotoh, 1997; Terzoudi et al., 2003; Malik et al., 2004) (2) karyotyping of chromosomes (Kowalska et al., 2003), (3) chromosome instability analysis (Bezrookove et al., 2003), (4) prenatal diagnosis (Srebnik et al., 2005), and (5) others (Johnson et al., 1999; Ito et al., 2002; El Achkar et al., 2005; Mochida et al., 2005; Someya et al., 2006).

CONCLUSIONS

PCC has been long considered a useful tool for studies of chromosome dynamics. The new technique of drug-induced PCC by protein phosphatase inhibitors has several advantages over the previous one based on cell fusion, and has lead to a wide spectrum of research activities in several laboratories, from basic cell biology to practical applications in radiation medicine. Despite decades of careful molecular studies, the detailed mechanism of chromosome condensation in normal cells entering mitosis is still not completely understood. It is likely that studies on "forced," premature condensation will also decisively contribute to understanding this normal, fundamental cellular process.

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