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Caffeine-Induced Uncoupling of Mitosis from the Completion of DNA Replication in Mammalian Cells

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Caffeine was shown to induce mitotic events in mammalian cells before DNA replication (S phase) was completed. Synchronized BHK cells that were arrested in early S phase underwent premature chromosome condensation, nuclear envelope breakdown, morphological "rounding up," and mitosis-specific phosphoprotein synthesis when they were exposed to caffeine. These mitotic responses occurred only after the cells had entered S phase and only while DNA synthesis was inhibited by more than 70 percent. Inhibitors of protein synthesis blocked these caffeine-induced events, while inhibitors of RNA synthesis had little effect. These results suggest that caffeine induces the translation or stabilizes the protein product (or products) of mitosis-related RNA that accumulates in S-phase cells when DNA replication is suppressed. The ability to chemically manipulate the onset of mitosis should be useful for studying the regulation of this event in mammalian cells.

IN YEAST AND MAMMALIAN CELLS, MITOSIS does not occur when S phase is interrupted by drug treatments or by restrictive conditions for mutants that are temperature-sensitive for DNA synthesis (*1*). Although premature chromosome condensation (PCC) and breakdown of the nuclear envelope can be induced in S-phase cells by fusing them with mitotic cells (*2, 3*), these events result from the transfer of preformed mitotic factors, not the uncoupling of endogenously induced mitotic events from the completion of DNA replication. The BHK (Syrian hamster fibroblast) temperature-sensitive mutant tsBN2 (*4, 5*), which undergoes PCC and other early mitotic events at the restrictive temperature, provides the only example of a cell line in which mitotic events can occur before S phase is completed. The biochemical processes that initiate mitosis are still unknown.

We have found that normal BHK cells that were arrested in S phase (*6*) and treated with caffeine underwent, within a few hours, the same early mitotic events described by Nishimoto (*4, 5*) for the tsBN2 mutant. Caffeine could induce PCC at con-

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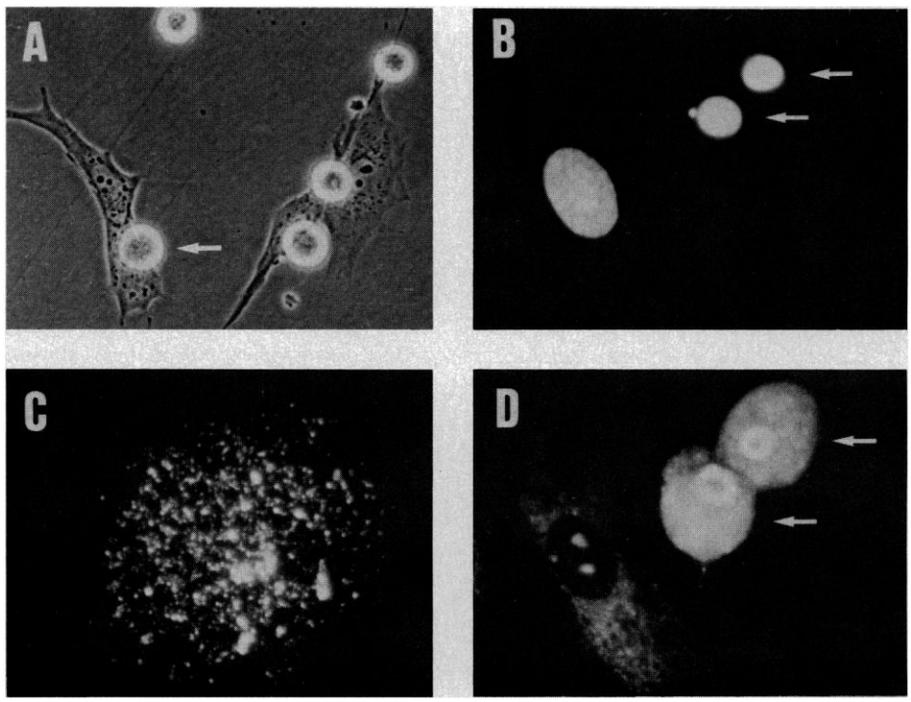

Fig. 1. Cytoplasmic, chromosomal, and phosphoprotein synthesis changes in PCC-containing cells (indicated by arrows). Cells were plated at 1×10^5 per 60-mm dish in Dulbecco's modified Eagle's medium (DME) + 10 percent fetal calf serum (FCS) and incubated for 24 hours. Synchrony was achieved by incubation in isoleucine-deficient DME + 5 percent dialyzed FCS for 34 hours (*16, 17*). Cells were then treated with DME + 10 percent FCS + 2.5 m*M* hydroxyurea for 14 hours, and finally were given the specified drugs ($T = 0$) along with Colcemid (0.3 μg/ml). (A) Phase contrast photograph (×840) of "rounded up" PCC-containing cells 4 hours after the addition of 5 m*M* caffeine. No "rounded up" cells were seen in untreated controls. (B) Condensed chromatin of cells 8 hours after the addition of 5 m*M* caffeine. Cells were fixed in absolute methanol and stained for 10 minutes with Hoechst 33242 (1 μg/ml) (×2100). (C) Chromosome preparation showing S-phase PCC stained with Hoechst 33242 (×2100). Four hours after the addition of 5 m*M* caffeine and Colcemid (0.3 μg/ml) the cells were trypsinized, treated for 20 minutes at 37°C in 0.55 percent KCl, fixed for 30 minutes in methanol:acetic acid (3:1), dropped on wetted slides, and heated at 70°C overnight before staining. (D) Indirect immunofluorescence of mitosis-specific phosphoproteins present in both the cytoplasm and chromatin of the same PCC-containing cells shown in (B). Monoclonal antibody MPM-2 (*9*) and rhodamine-conjugated, goat-antimouse secondary antibody were diluted 1:500 and 1:1000, respectively, in phosphate-buffered saline (PBS) containing 1 percent bovine serum albumin. Methanol-fixed cells were preincubated at 37°C with DME + 10 percent FCS for 30 minutes; incubated with MPM-2 for 4 hours; rinsed successively with DME + 10 percent FCS, PBS, and distilled water; air-dried; incubated with the fluorescent secondary antibody for 1 hour; and rinsed as before.

centrations of 200 μ*M* or above, with 1 m*M* being 60 percent as effective as 5 m*M*. The methylxanthines theobromine and theophylline were 70 percent and 20 percent as active as caffeine, respectively, when all compounds were assayed at 2 m*M*. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was inactive at doses up to 5 m*M*, indicating that phosphodiesterase inhibition was not solely responsible for these effects.

Caffeine-induced cytoplasmic "rounding up" and chromatin condensation of hydroxyurea-arrested cells are shown in Fig. 1, A and B, respectively. The chromosome preparation (*7*) in Fig. 1C illustrates that caffeine-induced PCC had the same "pulverized" appearance as that seen in PCC of S-phase cells that had been fused with mitotic cells (*8*). Mitosis-specific phosphoproteins were synthesized by these cells (Fig. 1D) as seen by indirect immunofluorescence with a monoclonal antibody that recognizes a family of such proteins (*9*). Autoradiography showed that RNA synthesis was inhibited in PCC-containing cells, as has been reported in normal mitotic cells (*10*). Thus, caffeine can induce cells that are arrested in S phase

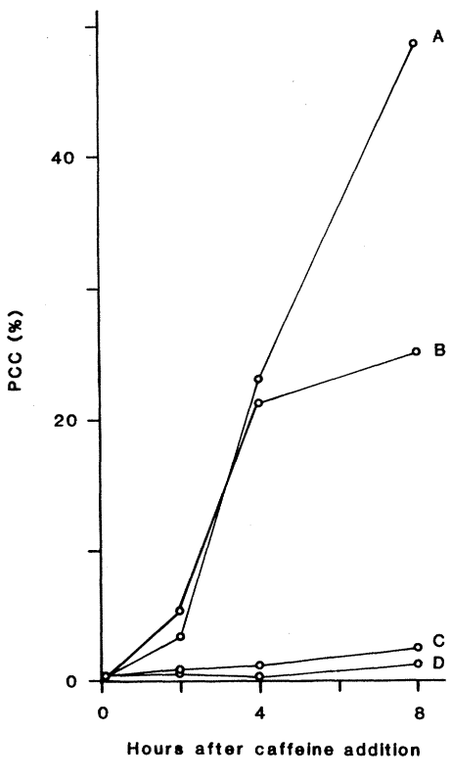

Fig. 2. Induction of PCC in cells that had been arrested in early S phase by (A) 5 m*M* caffeine; (B) 5 m*M* caffeine + actinomycin D (2 μg/ml); and (C) 5 m*M* caffeine + cycloheximide (1 μg/ml). (D) Untreated control. Cells were synchronized as described in Fig. 1. At each time point cells were fixed in absolute methanol and stained with Hoechst 33242 (1 μg/ml) for 10 minutes. At least 300 cells were examined at ×400 by fluorescent microscopy to determine the percentage of cells containing PCC.

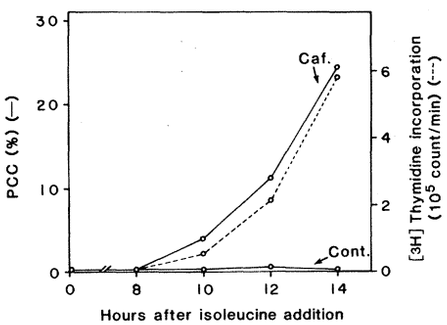

Fig. 3. Relationship of caffeine-induced PCC to the entrance of cells into S phase. Cells were released from isoleucine deprivation into DME + 10 percent FCS containing either [^{3}H]thymidine at 1 μCi/ml (group A) or 2.5 m*M* hydroxyurea (group B). Group A was analyzed for incorporation of [^{3}H]thymidine by precipitation in 10 percent trichloroacetic acid, followed by treatment with 0.2*N* NaOH and scintillation counting. Group B was treated at each time point with 5 m*M* caffeine (or PBS for controls), actinomycin D (2 μg/ml; to inhibit RNA synthesis during the 4-hour assay) and Colcemid (0.3 μg/ml). Four hours later, the cells were examined for PCC.

to undergo events that normally occur in early mitosis (*11*).

Caffeine-induced PCC required de novo protein but not de novo RNA synthesis (Fig. 2). These results were not appreciably altered when protein synthesis was inhibited by anisomysin (1 μg/ml) rather than cycloheximide or when RNA synthesis was inhibited by at 15 μg/ml DRB (5,6-dichloro-1-β-D-ribofuranosyl benzimidazole) rather than actinomycin D. The addition of transcription or translation inhibitors 30 minutes before caffeine exposure produced the same responses as when they were given simultaneously.

The following results suggest that the RNA needed for these mitotic events does not accumulate until the cells enter S phase. Cells made quiescent (G_0) by isoleucine deprivation did not display caffeine-induced PCC. When these cells were released from G_0, PCC could be induced by 5 m*M* caffeine in the presence of actinomycin D (2 μg/ml) only when treatment was started after DNA replication had begun, as measured by [^{3}H]thymidine incorporation (Fig. 3). Entry of cells into S phase has also been reported as a requirement for PCC induction at the restrictive temperature in the BHK mutant tsBN2 (*5*). In an additional experiment, wild-type BHK cells were released from isoleucine deprivation for 14 hours in the presence of [^{3}H]thymidine (1 μCi/ml) and 0.5 m*M* hydroxyurea (a dose that inhibited DNA synthesis by 88 percent but still permitted DNA labeling). When these cells were subsequently treated with 5 m*M* caffeine and actinomycin D (2 μg/ml) for 4 hours, autoradiography revealed that more than 99 percent of all PCC-containing cells had labeled DNA while only 35 percent of the unresponsive cells were labeled.

DNA replication in S-phase cells had to be strongly suppressed before caffeine could initiate PCC (Fig. 4). Significant induction of PCC required more than a 70 percent reduction in DNA synthesis and reductions of more than 98 percent were needed for maximal responses. Similar results were obtained with hydroxyurea and aphidicolin, implying that DNA synthesis inhibition rather than unrelated drug effects was essential for caffeine-induced PCC.

Caffeine can partially reverse the inhibition of DNA synthesis resulting from low doses of hydroxyurea (*12*) or aphidicolin (*13*). The PCC occurring in our experiments was not the consequence of caffeine overcoming the hydroxyurea or aphidicolin block, thereby permitting cells to complete S phase and subsequently enter mitosis, since (i) the morphology of the PCC's was typical of S-phase cells (Fig. 1C), (ii) caffeine-induced PCC did not occur in the absence of hydroxyurea or aphidicolin (Fig. 4), and (iii) 5 m*M* caffeine did not increase [^{3}H]thymidine incorporation during S-phase arrest with 2.5 m*M* hydroxyurea (*14*).

Our experiments suggest that RNA that is needed for mitosis accumulates either when DNA replication is suppressed or, as in untreated cells, when DNA replication is completed (*15*). Caffeine may subsequently induce the translation or stabilize the protein product(s) of this RNA. By modifying post-transcriptional control mechanisms, caffeine is able to uncouple mitotic events from the normal completion of DNA replication. Investigations of this post-transcriptional control should help in understanding the mechanism for signaling the onset of mitosis.

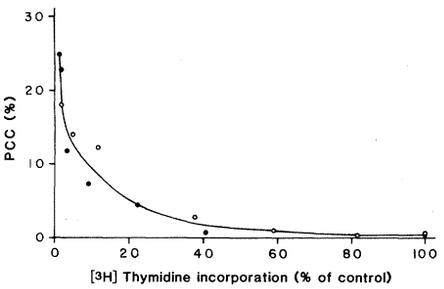

Fig. 4. Relationship of caffeine-induced PCC to the suppression of DNA synthesis. Cells were synchronized at early S as in Fig. 1. At T = 0, 2.5 m*M* hydroxyurea was removed and either (i) 0, 0.05, 0.1, 0.2, 0.5, 1.0, or 2.5 m*M* hydroxyurea or (ii) aphidicolin at 0.1, 0.2, 0.5, 1.0, 2.0, or 5.0 μg/ml was added back along with 5 m*M* caffeine and Colcemid (0.3 μg/ml). Four hours later, [^{3}H]thymidine incorporation and percent PCC were measured as described in Figs. 3 and 2, respectively. Hydroxyurea (○); aphidicolin (●).

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Visual Pigment Homologies Revealed by DNA Hybridization

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A bovine rhodopsin complementary DNA probe was used to detect homologous visual pigment genes in a variety of species. Under stringent DNA hybridization conditions, genomic DNA from most vertebrate species carried a single homologous fragment. Additional homologies were detected in some vertebrates by reducing the hybridization stringency. Homologous fragments were also detected in DNA isolated from invertebrate species, a unicellular alga, and an archaebacterium; many of these fragments were homologous to a *Drosophila* opsin probe. These results suggest that photosensory pigments in a wide variety of species arose from a common precursor.

VISUAL PIGMENTS ARE A CLASS OF receptor proteins that absorb light and trigger sensory signals. Rhodopsins, the visual pigments in vertebrate rod-type photoreceptor cells, consist of an intrinsic membrane protein (opsin) covalently linked to a vitamin A chromophore (*1*). Other classes of visual pigments, including cone-type photoreceptor pigments and invertebrate visual pigments, have been less well characterized biochemically because of their low abundance and instability. As an alternative to studying visual pigments at the protein level, the genes encoding these proteins can be identified, their sequences can be determined, and the comparative genetic information can be assessed. We explored the potential for using a bovine opsin complementary DNA (cDNA) probe to identify homologous genes in other species. Using genomic Southern blot hybridization (*2*), we probed the genomes of a variety of species in a manner analogous to that reported for other protein families (*3*). Our results demonstrate that bovine opsin has coding regions homologous with visual pigment genes of vertebrate, invertebrate, and phototactic unicellular species.

We surveyed a variety of vertebrate species for homologous visual pigment genes under stringent hybridization conditions that allow reannealing of the probe with only closely related sequences (*4*). Genomic DNA isolated (*5*) from human, mouse, cow, sheep, chicken, chameleon, gecko, frog, and goldfish tissues was prepared and hybridized by the method of Southern (*2*) with a radioactive cDNA probe (SP1116) containing the entire coding sequence for bovine opsin (Fig. 1a). Under these conditions, homologous DNA fragments were detected in most of the vertebrate DNA tested (Fig. 2a and Table 1). With one possible exception—goldfish—the homologous sequences were confined to a single restriction fragment in the pattern produced by at least one of the restriction endonucleases used. Such a hybridization pattern, combined with an assessment of the intensity of hybridization (lane 1 in Fig. 2a), suggests that the homologous sequences are present as single copies within their respective genomes. The hybridization patterns shown in Fig. 2a and Table 1 for the bovine and human DNA samples are entirely consistent with the restriction maps (*6*) for the corresponding opsin genes shown in Fig. 1, b and c. It is apparent that SP1116 hybridizes specifically to the rod visual pigment genes present as a single copy in these two species. We suggest that the homologies detected for the other vertebrate DNA under these stringent hybridization conditions also represent opsin genes with marked sequence conservation.

The two reptile species included in our survey, the gecko and chameleon, produced some unusual hybridization results. Hybridization of SP1116 to gecko DNA under stringent conditions gave no detectable signal, indicating a lack of strong homology between the gecko and bovine rod visual pigment genes (Table 1). The lack of hybridization to gecko DNA under these conditions cannot be attributed to a large genome size, as reptiles generally have genomes that are smaller than those of mammalian species (*7*). Although both the bovine and gecko retinas contain rod-type photoreceptor cells, gecko rods are unlike the rod cells of other species (*8*). It has been proposed that gecko rods evolved from cone-type photoreceptors by a process of transmutation, and there is corroborating physiological evidence that the gecko rod pigment is more like a cone pigment than an opsin (*8*). Such a hypothesis would explain our inability to detect opsin-like visual pigment genes in this species under stringent hybridization conditions. Conversely, the chameleon *Anolis carolinensis* is considered to lack rod-type photoreceptors altogether (*9*), yet our data show hybridization of SP1116 under stringent conditions (lane 7 in Fig. 2a). Thus we can detect the presence of a gene closely related to bovine opsin in an animal with an all-cone retina. Possible explanations are (i) that the gene encodes a cone visual pigment that is strongly homologous to the rod pigments of other animals, (ii) that the gene is not expressed in the retina, and (iii) that this animal does indeed possess a small population of rod photoreceptor cells that have escaped detection by other methods. A detailed molecular analysis should provide a clearer understanding of

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