The plasmodia of *Physarum polycephalum*, an elegant system to demonstrate the importance of genome integrity in traversing the G2/M checkpoint of the cell cycle

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In this communication we show the usefulness of the plasmodia of *Physarum polycephalum* to demonstrate important concepts in cell cycle regulation, such as the need for an undamaged chromosomal DNA to allow the transition from late G2 to prophase (G2/M checkpoint). In *Physarum*, chromosomal DNA synthesis is confined approximately to the first one-third of the cell cycle which comprises the S phase, and rDNA and mitochondrial DNA are synthesized throughout the cycle except during mitosis and early S phase. Taking advantage of these facts, DNA of the plasmodia was substituted with 5-bromo-2′-deoxyuridine at a concentration where it had no effect on DNA synthesis *per se* during different phases of the cell cycle before exposure to UV during late G2. A strong synergistic effect between BrdU and UV, in terms of mitotic delay, was observed only in plasmodia treated with the base analogue during the early S phase, thereby showing that the integrity of chromosomal DNA is an important prerequisite for the initiation of mitosis. It is suggested that this system is ideal for molecular level studies on this aspect of checkpoint operation.

IN eukaryotic cells, progression of cell cycle through different phases (G1, S, G2 and M) is controlled by a cell cycle control system. This system comprises brakes – the check points – that can stop the cell cycle at two specific points – one in G1 before entry into S phase (G1/S checkpoint) and another in late G2, at the entry into mitosis (G2/M checkpoint). The checkpoints are signal transduction pathways whose effectors interact with cyclin/Cdk complexes to block cell cycle progression. A large number of studies have been carried out in yeast and mammalian cells to understand the molecular mechanisms operating at these points[1–7].

Although the importance of chromosomal DNA in initiating mitosis[8,9], and that of G2 phase as a period of surveillance to monitor the state of the nuclear DNA, before chromosomes are allowed to participate in mitosis and another cycle of reproduction, was envisaged since some years[10], there has been a renewed interest now in understanding the mode of communication of chromosomal DNA with the signals which allow the cells to traverse the G2/M checkpoint. In the case of severe damage, G2 phase is also a time to activate programmed cell death[11–13].

Entry into mitosis requires at least two events: activation of a mitosis promoting protein called Cdc2 by the Cdc25 phosphatase; and accumulation of active Cdc2 in the nucleus. The DNA damage checkpoint seems to block both these processes via pathways dependent on p53, a tumour suppressor protein, firstly by maintaining Cdc25 phosphatase in a phosphorylated form by protein kinase Chk1, followed by the binding of various 14-3-3 proteins[14] and the resultant export of this phosphatase out of the nucleus, thus preventing the Cdc2–cyclinB complex from getting activated. The other pathway operates via 14-3-3σ protein which binds Cdc2–cyclin complex and sequesters it in the cytoplasm. There is evidence for the involvement of yet other factors and there could also be some amount of redundancy in the mechanisms operating at this checkpoint and further investigations are required to elucidate them[15,16]. Recent studies on cell-free extracts of *Xenopus* eggs which analysed the role of protein kinase Chk1 in checkpoint control show the similarity of this system with that of yeast and mammals[14]. There is also the proposal by Lydall and Weinert[13], that damaged DNA could structurally inhibit mitosis, i.e. if condensed chromatin was a structural prerequisite for entry into mitosis, and damaged DNA could not be efficiently condensed.

Different types of perturbers have been used to analyse cell cycle regulation in the synchronously mitotic plasmodia of *Physarum polycephalum*[17]. It is known that UV irradiation during G2 inhibits RNA and protein syntheses[18], and interferes with the organization of microtubular organizing centres[19,20] in this organism. Irradiation also leads to long mitotic delays[18,21]. It had also been observed that UV-irradiation causes decondensation of chromosomes in the plasmodia[22]. The higher susceptibility of S phase to UV in the plasmodia is also known[21]. Therefore, to single out the mitotic delaying effect of UV at the level of the integrity of chromosomal DNA *per se*, it is necessary to irradiate the plasmodia during very late G2, when chromosomal DNA synthesis is already completed and the chromosomes are condensed. It would also be ideal to use plasmodia whose DNA has already been sensitized to UV such that any effect is amplified.

The analogue of thymidine, 5-bromo-2′-deoxyuridine [BrdU] is readily incorporated into the DNA of the cells including that of the plasmodia of *Physarum*[23]. In the present experiments, it was thought desirable to accentuate the effect of UV on DNA by substituting it with this analogue, as BrdU incorporated DNA is known to be highly sensitive to radiation[24]. Along with BrdU, 5-fluorodeoxyuridine (FudR) and uridine (UR) were also supplied in the ratio 8 : 1 : 20, as was done earlier by McCormick *et al.*[25]. They had shown that FudR given in this ratio to the plasmodia of *Physarum* along with BrdU, would increase the

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incorporation of BrdU, into the DNA, because of FudR's known ability to block thymidine synthesis. UR in the above proportion was sufficient to prevent a decrease in the rate of RNA synthesis, caused by a breakdown of FudR to 5-fluorouracil and the consequent inhibition of UR biosynthesis. In the plasmodia of *Physarum* the chromosomal DNA synthesis is confined to the first one-third of the cell cycle which comprises the S phase (the G1 being absent). The cycle time varies from 8 to 12 h and the replication of the other DNA species (ribosomal DNA and mitochondrial DNA) takes place throughout the cycle except during mitosis and the early part of S phase[26,27]. In order to distinguish between the different DNA types, BrdU treatment was carried out at different phases of the cell cycle in surface plasmodia prepared as described below.

The McArdle strain (M3C) of *P. polycephalum* was grown as microplasmodia in shaken cultures in the dark at 24°C on a semi-defined medium (SDM)[28]. Mitotically synchronous surface (macro) plasmodia were prepared by the fusion of microplasmodia (0.4 ml packed suspension) on Whatman No. 40 filter paper[29] and grown in petri dishes supplied with SDM. Thereafter at regular intervals, synchronous postfusion mitoses (PFM) occur in these plasmodia.

Mitotic delay studies were carried out after irradiating (dose: 1400 Jm^{-2}), both the BrdU-substituted (at different phases of the cell cycle) and unsubstituted plasmodial sectors at late G2 prior to the third PFM (details are given in legend to Figure 1), using a Philips 15 W germicidal lamp which had earlier been calibrated (dose rate: 7.18 $Jm^{-2} s^{-1}$) by actinometry[30], and which emits approximately 90% of its UV energy at 2537 Å. DNA synthesis was evaluated by the radioisotope dilution technique of Devi and Guttes[31] (details are given in the legend to Figure 2), in the case of plasmodia substituted with BrdU during early S phase, where alone enhanced mitotic delay was observed.

The synergistic effect of UV and BrdU on mitotic delay was observed only in early S phase analogue-substituted category of plasmodia, in spite of increasing the treatment doses to 20 $\mu g\ ml^{-1}$ during late S phase or to 40 $\mu g\ ml^{-1}$ thereafter (Figure 1). Since mitochondrial and nucleolar DNA synthesis is seen throughout the cell cycle except during early S phase and mitosis[26,27], the mitotic delay data (Figure 1) clearly indicate that BrdU incorporated into the chromosomal DNA of *Physarum* during the S phase of the cell cycle is the cause for the observed enhanced delay in the UV-irradiated system. The incorporated BrdU as such also causes some delays, particularly in those plasmodia treated with this analogue during early S phase.

The extra sensitivity seen in the plasmodia treated with BrdU during the early part of the S phase (significantly more than that seen with late S phase treatments) is understandable in view of the reported master-initiation of majority of the chromosomal replicons towards the early part of the S phase and the increasing distances between regions in actual replication at later times in the S phase in the plasmodia of *Physarum*[23]. In fact, it is known that approximately 80% of the nuclear DNA of the plasmodia is replicated during the first 1 h 30 min of the S phase and much of the remaining 20% during the latter part of the S phase[26]. This way a higher rate of incorporation of BrdU into the chromosomal DNA is expected during early S phase.

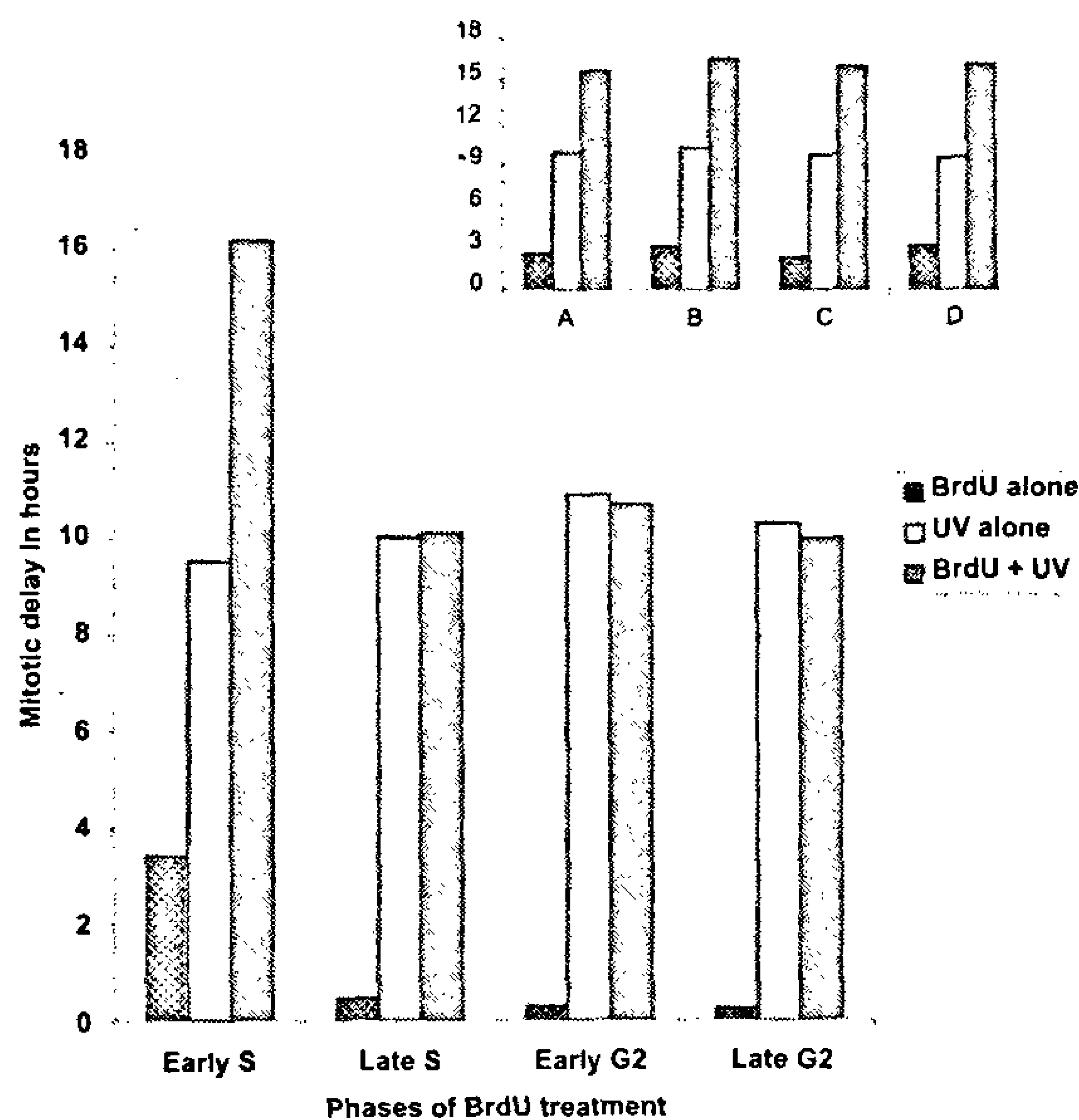

Figure 1. Effect of late G2, UV-irradiation on the mitotic cycle of BrdU substituted (at different phases of the cell cycle) plasmodia of *Physarum polycephalum.*

Each of the plasmodium in a set of sister plasmodia, made from pooled microplasmodial suspension, was cut into 4 sectors at the metaphase of the second PFM, and immediately after this, two of the sectors (sectors are synchronous with respect to each other) were pulse-treated for 2 h, during early S phase, with BrdU (5 $\mu g\ ml^{-1}$ of SDM). After the BrdU pulse and serial changes in fresh SDM to wash off the unincorporated analogue, both the control and the BrdU-substituted sectors were grown on fresh SDM till late G2. At approximately 30 min before their respective third PFM, one control (BrdU unsubstituted) and one BrdU-substituted sector from each plasmodium were UV-irradiated. The time of mitosis in all the sectors was noted and the mitotic delay due to the perturbations (i.e. BrdU alone, UV alone and BrdU + UV) was calculated with respect to their respective control sector (untreated and unirradiated).

Keeping the basic scheme of the experiment as above, 2 h BrdU pulse treatment was given during other phases of the cell cycle (late S, early G2 and late G2), followed by irradiation in late G2. In all these cases the mitotic delay due to the perturbations was calculated with respect to the respective control. Since 5 $\mu g\ ml^{-1}$ of BrdU was found to have no mitotic delaying effect when supplied during late S and G2 phases, still higher concentrations (20 and 40 $\mu g\ ml^{-1}$) were used for these phases.

Values obtained from two plasmodia of each category have been averaged out and plotted in the bar diagram (Figure 1). Inset shows a projection of the values from four different plasmodia of the early S phase category, to highlight the extra sensitivity obtained here.

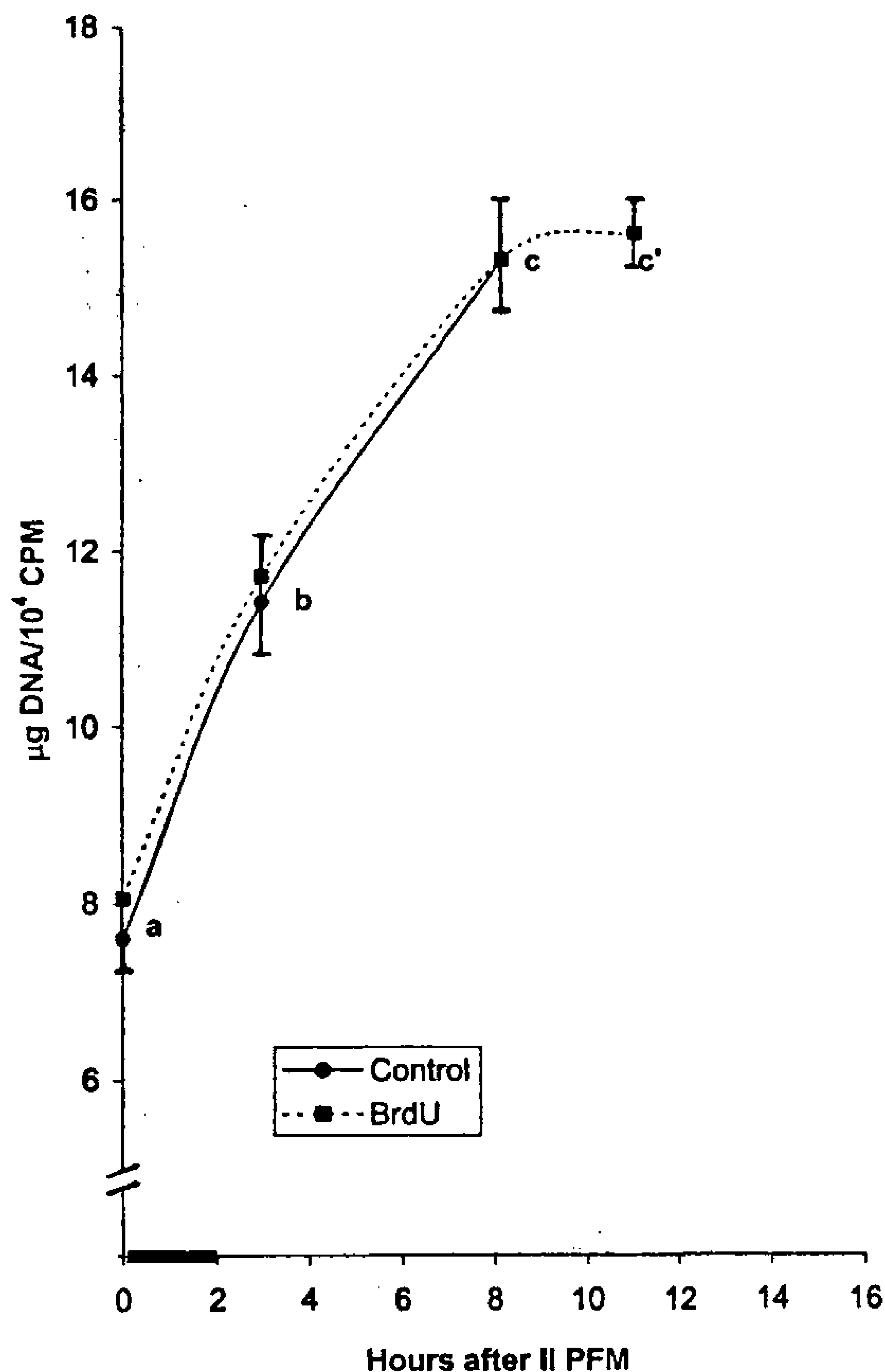

Figure 2. Effect of 5 µg ml^{-1} BrdU incorporation during early S phase on DNA synthesis in the plasmodia of *Physarum polycephalum*. The bar on the abscissa indicates the time and duration of BrdU treatment in the treated category. The vertical bars (I) on the points denote the range of values.

48-h-old microplasmodial cultures were labelled for 24 h with ^{3}H-thymidine (5 µCi ml^{-1} of SDM; sp. activity 18.5 Ci/m mole; BARC, India). At the end of this period, the microplasmodia were washed free off the radioisotope and were used for making surface plasmodia. The surface plasmodia were then grown on label-free medium, and were transferred to SDM containing BrdU (5 µg ml^{-1}) for 2 h on citing the prophase of the second PFM. At the prophase of the second PFM and at subsequent intervals till the prophase of the third PFM, samples were collected from these plasmodia for determining the specific activity of the DNA (cpm/µg DNA) in them. The dilution of the label here then would indicate the amount of DNA synthesis in these plasmodia. For convenience in plotting, we have shown this as increase in DNA for a given value of cpm (µg DNA/10^4 cpm). This method[31] was adopted for analysing DNA synthesis, as routine pulse-labelling with ^{3}H-thymidine during S phase is not possible here because of the BrdU treatment during this period. DNA was extracted and estimated by the method of Mittermayer *et al.*[36], and aliquots from the extract were spotted on Whatman GF/C filters and counted in a LKB RackBeta LSC using a toluene, PPO, POPOP cocktail.

Values obtained from three plasmodia have been averaged out and plotted in Figure 2, showing also the range of variations. Samples were collected at (a) the prophase of second PFM, (b) 3 h after second PFM, (c) the prophase of third PFM in control and (c′) the prophase of third PFM in BrdU-substituted.

The enhancement of the G2-phase, UV-induced mitotic delay, because of S phase incorporation of Brdu into the chromosomal DNA of the plasmodia, is in agreement with the known extra vulnerability of BrdU-substituted DNA to UV[32], apparently because of the production of DNA strand breaks and also DNA–DNA and DNA–protein cross-linkings in such systems[33,34]. Although G2-phase UV-irradiation as such is highly inhibitory to RNA and protein synthesis[18], it is known from earlier studies that additional inhibitions were not observed because of BrdU incorporation[35]. At the concentration (5 µg ml^{-1}) used to obtain extra mitotic delay, DNA synthesis was also not affected (Figure 2). As noted earlier, there was no enhancement in late G2, UV-induced mitotic delay, when BrdU treatment was carried out during the other phases of the cell cycle (Figure 1). The results of the present experiments clearly show that UV-induced alterations of chromosomal DNA, as late as G2-prophase transition period, can inhibit and postpone mitosis. This could well be because of the need for a signal based on the status of chromosomal DNA (undamaged) to start the process of mitosis[8,9,12,13]. It is already known that the DNA synthetic phase in the plasmodia is highly susceptible to UV-irradiation and maximum mitotic delay is obtained when irradiated during early S phase[21]. However, in this study we have been able to show the importance of the integrity of chromosomal DNA as such, in initiating the process of mitosis in this organism, which is quite independent of DNA synthesis. The idea behind these experiments was to show the elegance of the experimental system for demonstrations of this nature, and to suggest that the natural synchrony obtained here could be utilized in future molecular level investigations on checkpoints linking chromosomal DNA to G2/M transitions.

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Agrobacterium-mediated genetic transformation and regeneration of transgenic plants from cotyledon explants of groundnut (*Arachis hypogaea* L.) via somatic embryogenesis

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An efficient transformation protocol was developed for groundnut (*Arachis hypogaea* L.) plants. Precultured cotyledons were co-cultured with *Agrobacterium tumefaciens* strain LBA 4404 harbouring the binary vector pBI121 containing the *uidA* (GUS) and *nptII* genes for 2 days and cultured on an embryo induction medium containing 0.5 mg/l NAA, 5.0 mg/l BAP, 75 μg/ml kanamycin and 300 μg/ml cefotaxime. The putatively transformed embryos were transferred to the medium with reduced kanamycin (50 μg/ml) for further development. Prolific shoots developed from these embryos on a MS medium containing 0.5 mg/l BAP and 50 μg/ml kanamycin with a transformation efficiency of 47%. The elongated kanamycin-resistant shoots were subsequently rooted on the MS medium supplemented with 1.0 mg/l IBA. The transgenic plants were later established in plastic cups. A strong GUS activity was detected in the putatively transformed plants by histochemical assay. Transformation was confirmed by PCR analyses. Integration of T-DNA into nuclear genome of transgenic plants was further confirmed by Southern hybridization with *nptII* gene probe. A large number of transgenic plants were obtained in this study. This protocol allows effective transformation and quick regeneration via embryogenesis.

GROUNDNUT or peanut (*Arachis hypogaea* L.) is one of the principal economic oilseed legumes and is largely cultivated in many tropical and subtropical regions of the world. The seeds are mostly used to supply vegetable oil, carbohydrates and proteins for human as well as animal consumption. Crop improvement by conventional breeding in this important oilseed crop is not as rapid as envisaged to meet the demands of increasing population, especially in seed quality improvement and developing virus- and insect-resistant varieties. There is an urgent need to improve several commercially grown varieties in India and elsewhere. Tools of genetic engineering can be exploited as an additional method for introduction of agronomically useful traits into established cultivars. Major seed proteins of groundnut as well as of other leguminous

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