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Abstract

Experimentation was performed primarily to determine whether progression of the DNA replication fork along segments of S phase *Xenopus* chromosomes, which contain UV-induced pre-aberrational lesions, plays a significant role in conversion of these lesions into chromatid deletions. Specifically, a *Xenopus* chromosome that was both easy to identify and that possessed a single DNA replication fork in one arm was found and used to conduct the experimentation. This chromosome was exposed to UV in early S phase and a Bromodeoxyuridine/Giemsa differential staining technique was applied in conjunction with conventional aberrational techniques to correlate progression of the DNA replication fork through segments of this arm with chromatid deletion production in these segments. The results point to "direct" evidence for the role of the DNA replication fork in converting some UV-induced pre-aberrational DNA damage into chromosomal deletions.

Introduction

Earlier studies of UV-induced chromosomal aberration production and related repair in interphase *Xenopus* tissue culture cells (Griggs and Bender, 1972; Bender et al., 1973; Griggs and Orr, 1979; Griggs and Payne, 1981;) have yielded essentially two key results. First, low UV (254 nm) fluences administered to interphase cells induced lesions that, if not repaired, led to a significant frequency of chromosomal aberrations observable at the first mitosis following the exposures. Second, when cells were exposed to relatively low fluences of UV in early G1 phase and then exposed to photoreactiveable light as a function of time, most of the UV-induced aberrational damage was photoreactivable through G1 phase, but as the cells entered S phase, their photoreactive capacity ceased. The intracellular process responsible for this alteration in photoeactiveability as the *Xenopus* cells enter S phase has not yet been properly elucidated. Currently, we are in the process of performing experimentation designed primarily for this purpose. Since DNA replication is a predominant cellular activity during S phase, an attempt to obtain "direct" evidence for the role of DNA replication in the process would appear to be the indicated initial effort.

The rationale for our initial experimentation was based on the following. Since it has been shown that the only photoreactivable aberrational damage in DNA is pyrimidine dimmers (Smith and Hanawalt, 1969), it is reasonable to expect that dimmers might distort the normal DNA configuration in such a manner as to interfere with the natural functioning of the DNA replication fork and associated mechanisms. Thia altered functioning might then be responsible for producing DNA breaks. A significant fraction of these breaks would lead to chromatid deletions observable at the first succeeding mitosis. Consequently, it should be possible to observe a definite correlation between the chromatid deletion positions on the chromosomes and the DNA replication fork position on the chromosomes if a technique were available for determining the positions of the DNA replication forks in chromosomes as a function of time during the replication of extended segments of the S phase chromosomes. Such a correlation would constitute "direct" evidence for an integral role of the DNA replication fork in (at least some) categories of chromosomal aberration production. Therefore, we report here an attempt to develop appropriate techniques to accomplish this and to describe the proposed correlation.

Materials and Methods

Cell line used, routine maintenance.--All experiments were conducted using the A87 *Xenopus* cell line that was cloned from the A8W243 line described by Griggs and Bender (1972). This line is maintained in the dark at approximately 23 degrees Celsius. Monolayers were present in large plastic bottles (Falcon) in F10 medium. This medium was supplemented with a 10% fetal calf serum solution (Hazleton) and buffered with $NaHCO_3$ (0.065 M) and HEPES (0.01 M) (Calbiochem.).

At this temperature in exponential growth, these cells usually exhibit a plating efficiency of at least 85%. The

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average cell cycle time was 52 hours, including 12 hours for G1, 30 hours for S, and 10 hours for G2 and mitosis. The cell line, which contains cells that possess 36 easily identifiable chromosomes, provided adequate cytological material for chromosomal analysis, including deletion detection.

A more detailed description of this cell line and these procedures can be found in previous publications (Griggs and Bender, 1972; Griggs and Orr, 1979; Griggs and Payne, 1981; Kulp and Griggs, 1989; Laswell et al., 1991). Techniques employed for mitotic index determinations, colony assays, cell plating, cell synchronizations, mitotic arrest, survival curve analysis, and chromosome spreads did not differ significantly from the procedures outlined in the publications mentioned above.

Bromodeoxyuridine (BrdU) labeling.--A 5-bromodeoxyuridine (BrdU) solution (5 by 10^{-4} M) /Giemsa differential staining method was used in this experiment to determine the presence and location of any DNA replication forks. Synchronized momolayers of cells were allowed to progress through S phase in the BrdU medium. Then, after the cells had entered the first mitosis (M1), they were stained with Giemsa as described by Perry and Wolff (1974). Mitotic selection at M1 produced cultures of unifiliarly labeled cells.

Flash labeling experiments were also conducted in connection with the procedure described above. Synchronized cultures were placed in BrdU medium at two hour intervals that spanned the entire S phase. Beginning at 10 hours after shake off, different synchronized cultures were placed in the BrdU medium for 25 minutes every two hours ending at 42 hours after shake off. The labeled portion of the chromatid in the flash labeling procedure appears as only a small section of the entire chromatid (as opposed to continuous labeling where the entire chromatid is labeled) and allows for a more accurate determination of the presence of different replication forks and their location throughout S phase.

A similar labeling procedure was developed by Perry and Wolf (1974) who described the essentials of this technique in more detail. Also, similar work was conducted by Laswell et al. (1991) who give a complete discussion of the metaphase spread techniques employed in this experiment.

UV irradiation.--The source of the UV-irradiation was composed of four 15-W germicidal lamps (Sylvania G15T8) mounted at the top of a small cabinet. Irradiation of the monolayers occurred at 254 nm using thin sheets of plastic between the lamps and the sample radiation surface for does rate control. Techniques did not differ significantly from those described in greater detail by Griggs and Orr (1979).

Results and Discussion

The determination of which chromosome or chromosomes would be appropriately suited for our experimentation rested on the following two conditions: (1) the chromosome must have been easily identifiable and distinguishable in a chromosome spread, and (2) to facilitate ease of data interpretation, the chromosome would preferably contain a chromatid with a single replication fork that replicated an extended section of DNA. A chromosome that met the criteria was found in the cells of the A87 *Xenopus* cell line.

First, the chromosome we used was easy to identify. It is easily distinguishable because one arm projecting from the centromere is normal and the other two arms are not - they are both shorter and appear to have undergone abnormal supercoiling. Second, within the limits of resolution of the labeling we used, the chromosome appeared to possess a single DNA replication fork that replicated all the DNA of the normal chromatid, beginning at the centromere and ending near the telomere (Fig. 1).

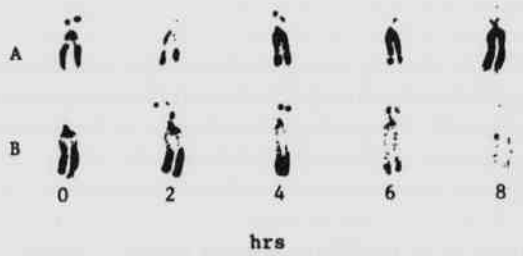

Fig. 1. BrdU labeled *Xenopus* chromosome at two hour intervals during S phase (numbers correspond to hours after S phase has begun). Set A contains the chromosome when flash labeled. Set B contains the chromosome when continuous labeling was employed. The chromosome exhibited a labeling pattern that indicated the presence of only one DNA replication fork that began at the centromere and ended near the telomere.

In the process of examining whether the chromosome met the second criterium, an accurate measure of the *Xenopus* cell cycle was determined - especially in regard to S phase when the DNA replication fork is present. Figure 2 contains data relating the BrdU labeling time with the number of chromosomes labeled at the following mitosis. Because BrdU is only incorporated into DNA during S phase when the DNA replication fork is present and synthesizing new DNA, only chromosomes that were in S phase during the flash labeling will appear labeled at M1. Therefore, from Fig. 2, it is readily apparent that S phase begins approximately 12 hours after shake off and appears to last until approximately 44 hours after shake

off, as is evidenced by the drop in labeled cells at M1 at this time (i.e., G2 is beginning here).

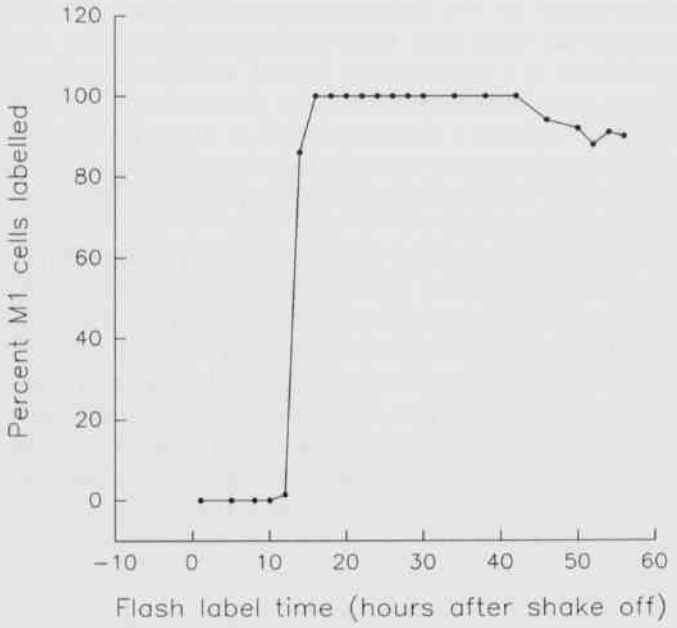

Fig. 2. Flash labeling curve to determine the duration of the cell cycle phases for synchronized G1 *Xenopus* cells. As indicated by the sharp rise in labeled cells, S phase begins at approximately 12 hours after shake off.

In addition to elucidating the S phase duration, it was necessary to our experiment to choose a proper UV fluency that produced sufficient damage to the chromosomes. However, it was also important that this UV fluency did not cause excessive damage which would possibly interfere with or prevent the DNA replication fork from interacting with the lesions. Figure 3 shows the effect of various UV fluences on both cell cycle time and mitotic index. The graph indicates that the cell cycle time is lengthened with increased UV fluency and that the mitotic index decreases with increased UV fluency, possibly because the repair mechanisms need additional time to carry out their functions (increased cell cycle time), and with more damage there is a greater chance that the cells will never be fully repaired and capable of entering mitosis (decreased mitotic index).

Based on Fig. 3, we felt the fluency that would best be suited for our experiment would be 9 J/m² - it would produce an adequate amount of damage, yet it would not cause so much damage that the interaction of the DNA replication fork with the lesions produced would be significantly altered or impaired. Figure 4 supports this assertion. Figure 4 is intimately related to Fig. 2. However, Fig. 4 plots the BrdU flash label time versus the percentage of labeled cells at M1 when 9 J/m2 was

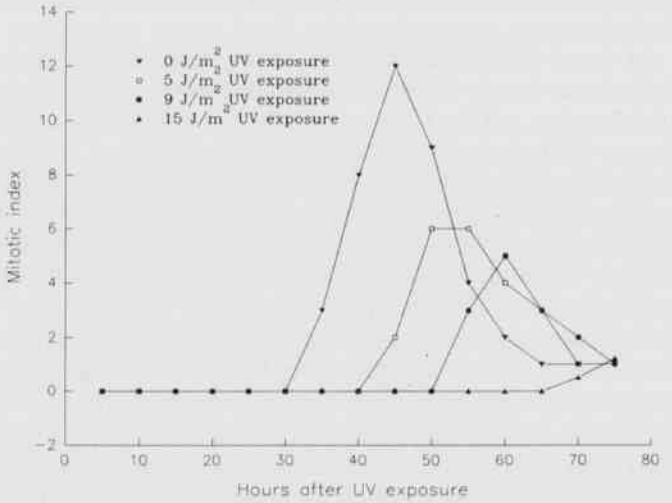

Fig. 3. Time course curve to determine the effect of various UV fluences on *Xenopus* cell cycle duration and mitotic index. UV radiation was administered at the onset of S phase (12 hours after shake off) at the indicated fluences.

administered at 12 hours after shake off, or the beginning of S phase. In contrast to Fig. 2, the cells represented in Fig. 4 were still in S phase even up to 70 hours after shake off. The best conclusion for the increased time spent in S phase is that intracellular repair mechanisms were functioning and required additional time to act. Thus, this data indicate there was indeed significant damage to the chromosomes caused by the 9 J/m² fluency used.

Figure 5 provides the data that ultimately led to our conclusion about the involvement of the DNA replication fork progression in converting the UV-induced lesions into chromosomal deletions. Figure 5 is a plot of the time at which the UV fluency of 9 J/m² was administered versus the percentage of chromosomes that possessed deletion at M1. The trend is obvious - the percentage of cells expressing deletions is indirectly proportional to the time at which the UV fluency was given. To best analyze Fig. 5, it is necessary to choose several points on the graph and describe in detail what we postulate is occurring.

Point A corresponds to the UV fluency given at approximately 12 hours after shake off where S phase is just beginning and the DNA replication fork is at its nearest to the centromere. When the UV fluency is administered, random lesions are produced all along the chromatid. Now, as the DNA replication fork moves down the chromatid toward the telomere, it has the highest probability of encountering the most number of lesions (i.e., it

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interacts with the maximum amount of irradiated DNA that is possible for it to encounter). If the DNA replica

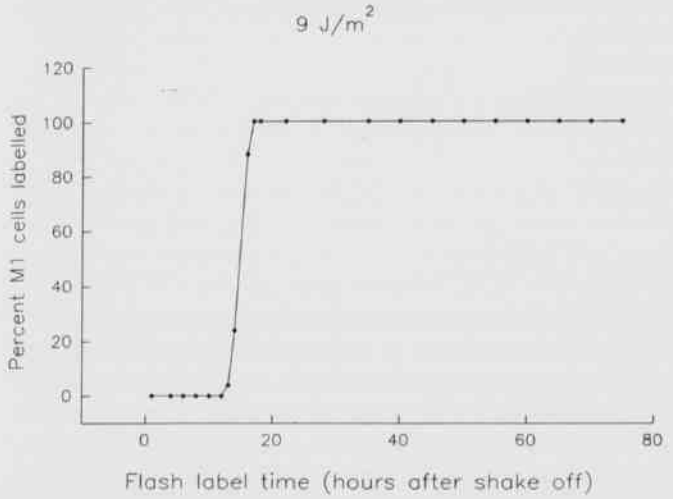

Fig. 4. Flash labeling curve to determine the duration of the cell cycle phases of synchronized G1 phase *Xenopus* cells following exposure to a 9 J/m² UV fluency administered at the onset of S phase (12 hours after shake off).

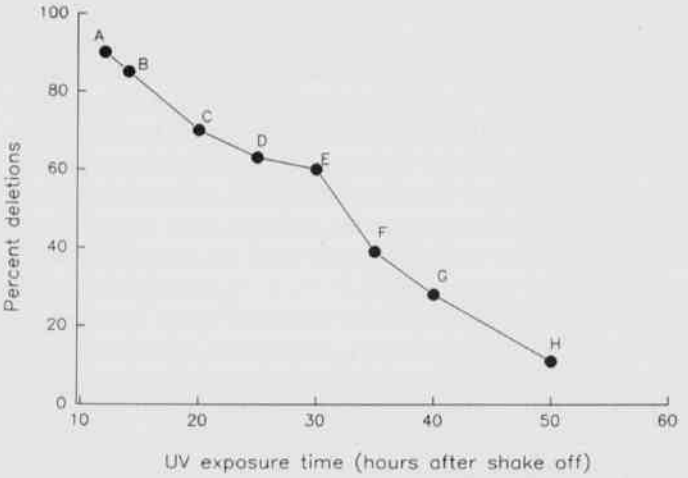

Fig. 5. Percentage of synchronized G1 phase *Xenopus* cells exhibiting chromatid deletions at M1 versus time of UV fluency (9 J/m²) administrations.

tion fork is involved in converting these UV-induced lesions into deletions, then point A should have the highest percentage of deletions - as it does.

Point E corresponds to the group of cells that were irradiated at approximately 30 hours after shake off. This is roughly half-way through the S phase. For illustrative purposes it is assumed that the DNA replication fork is essentially half-way through its trek along the chromatid when the UV fluency is given. Again, random lesions are produced all along the chromatid. However, in this case the DNA replication fork will encounter a lesser amount of irradiated DNA than points A, B, C, and D did. Thus, at point E the DNA replication fork has a lower probability of encountering as many lesions as it does at points A through D. Therefore, as stated above, if the DNA replication fork does play a role in converting these lesions into deletions, then point E should have a lower percentage of deletions than points A through D (and, using the same logic, point E should have a higher percentage of deletions than points F, G, and H) - as it does.

Finally, point H represents the group of cells that were irradiated when the DNA replication fork was at its closest point to the telomere. Here the DNA replication fork would interact with the least amount of DNA after UV exposure. Again, if the DNA replication fork is involved in the lesion to deletion process, point H should have the lowest percentage of deletions - as it does.

The data from a number of previous studies of UV-induced aberration production in interphase eukaryotic cells (Griggs and Bender, 1972; Wolff, 1972; Bender et al., 1973; Griggs and Payne, 1981) have been interpreted as indicating that dark radiation repair mechanisms, similar to excision or post-replication repair in *E. coli* (Rupp and Howard-Flanders, 1968; Walker et al., 1985) are intricately involved in aberrational processes. The essence of this interpretation is the idea that as damaged cells progress through the cell cycle, the repair mechanisms attempt to remove or circumvent the damage in various ways. The mechanisms are not 100% efficient and a number of aberrant structures result, including chromatid deletions, that appear with significant frequencies. The correlation between the DNA replication fork locations and the deletion frequencies at these locations suggests that some form of post-replication repair is more likely to play a significant role in deletion production than some type of excision repair. Post-replication repair is closely correlated with the replication fork and functions exclusively in S phase on portions of chromosomes that have already been replicated. On the other hand, excision repair is more of a random process that appears to function independently of the replication fork and in all phases of the cell cycle, not just in S phase (Walker et al., 1985).

In summary, the data described herein constitute "direct" evidence that the DNA replication fork is intricately involved in the conversion of some UV-induced lesions into chromatid deletions. Furthermore, these data, coupled with the results of earlier studies mentioned above, support the assertion that as the DNA replication fork attempts to replicate a section of DNA containing one of these lesions, an aberrant structure results,

which, if not corrected by post-replication repair, ultimately leads to a chromatid deletion.

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