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UV micro-irradiation of the Chinese hamster cell nucleus and caffeine post-treatment

Immunocytochemical localization of DNA photolesions in cells with partial and generalized chromosome shattering

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Summary

UV micro-irradiation of a small part of the Chinese hamster nucleus and caffeine post-incubation often results in shattered chromosomes at the first post-irradiation mitosis. In some of these mitotic cells, chromosome shattering is restricted to a few chromosomes spatially related in a small area of the metaphase spread; in others, shattering includes the whole chromosome complement. These 2 types of damage have been called partial and generalized chromosome shattering (PCS and GCS).

Using antisera that specifically react with UV-irradiated DNA, we identified micro-irradiated chromatin in interphase nuclei and in mitotic cells with PCS or GCS by indirect immunofluorescence microscopy. In PCS, immunofluorescence staining was found in the damaged area, while the surrounding intact chromosomes were not stained. In GCS, staining was also restricted to a small region of the shattered chromosome complement. In other experiments, cells synchronized in G1 were micro-irradiated in the nucleus, pulse-labelled with [^{3}H]thymidine and post-incubated with caffeine. Autoradiographs of cells with GCS showed unscheduled DNA synthesis restricted to a small chromatin region.

Our data present direct evidence that the distribution of DNA photolesions does not coincide with the sites of chromosomal damage in GCS. As a working hypothe-

sis, we propose that an indirect mechanism is involved in the induction of GCS by which DNA photolesions in a small nuclear segment induce shattering of both micro-irradiated and non-irradiated chromosomes.

In a number of cell strains, especially in rodent cells, caffeine is known to potentiate the chromosome-damaging effects of UV radiation and a number of chemical mutagens. (For a review see Kihlman, 1977.) A striking phenomenon observed after whole-cell UV irradiation (254 nm) and caffeine post-treatment is the frequent occurrence of cells with generalized chromosome shattering (GCS; fragmentation and/or pulverization of all chromosomes of a mitotic cell; Nilsson and Lehmann, 1975). This effect has recently been elucidated in more detail by C. Cremer et al. (1981) who found that GCS was induced by whole-cell irradiation of Chinese hamster cells at the first post-irradiation mitosis when the UV fluence exceeded a 'threshold' value in the sensitive phases of the cell cycle (G1 and S). These results and the finding of antagonistic effects of deoxyribonucleosides (C. Cremer et al., 1980a) are consistent with the view that DNA photolesions play a major role in the induction of GCS.

The method of laser-UV-micro-irradiation (257 nm; C. Cremer et al., 1974, 1976) has been used to show (Zorn et al., 1976; T. Cremer et al., 1980, 1981; C. Cremer et al., 1981) that chromosome shattering may also be induced by micro-irradiation of a small part of the interphase nucleus of Chinese hamster cells and caffeine post-treatment.

Interestingly, shattering was restricted to a few spatially related chromosomes in some mitotic cells, but involved all chromosomes in others (C. Cremer et al., 1981). We have referred to these 2 levels of chromosome damage as partial (PCS) and generalized chromosome shattering (GCS). Experimental conditions to induce PCS and GCS have been described in detail (C. Cremer et al., 1981). We suggested (1) that shattered chromosomes seen in mitotic cells with PCS reflect micro-irradiated chromosomes, whereas the intact chromosomes reflect non-irradiated ones and (2) that GCS indicates an effect where both micro-irradiated and non-irradiated chromosomes participate in shattering. To prove (or disprove) these 2 suggestions it is necessary to identify the micro-irradiated chromatin and to compare its distribution with the distribution of shattered and non-shattered chromosomes in single mitotic cells. Such an approach has become feasible by the use of antibodies that bind specifically to UV-irradiated DNA (Fukuda et al., 1976; Cornelis et al., 1977). Micro-irradiated chromatin can then be visualized by indirect immunofluorescence microscopy (C. Cremer et al., 1980b). A second approach takes advantage of unscheduled DNA synthesis induced in the micro-irradiated nuclear part. Micro-irradiated chromatin can be labelled by a pulse of [^{3}H]thymidine and identified by autoradiography (C. Cremer et al., 1979; Zorn et al., 1979; T. Cremer et al., 1982).

In the present investigation, we show that indirect immunofluorescence is restricted to shattered chromosomal sites in PCS. In GCS, both indirect immunofluorescence and unscheduled DNA synthesis are detected in a small part of the shattered chromosome complement only.

Materials and methods

Cell material

Cell cultures from the M3-1 Chinese hamster cell line (Gray et al., 1975) and from a fibroblastoid strain 'CHL' (Zorn et al., 1976, 1979) were grown in minimal essential medium supplemented with non-essential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% foetal calf serum (FCS) in a humidified atmosphere with 5% CO_2. For micro-irradiation experiments, cells were grown in plastic petri dishes (Nunc Ø 5 cm). Before inoculation of cells, 'experimental' fields of about 0.25 mm^2 were marked by scalpel cuts on the bottoms of the dishes. Asynchronously growing M3-1 cells were used for micro-irradiation 1 day after sub-cultivation of sub-confluent cultures. Synchronized CHL cells were obtained by shaking off mitotic cells from growing cultures. Mitotic cells were seeded in dishes with MEM containing 0.5% FCS to keep them in the G1 phase of the cell cycle for a couple of hours (Zorn et al., 1979).

Micro-irradiation procedure

Petri dishes were placed in a special irradiation chamber (C. Cremer et al., 1976) and cells growing in 'experimental fields' were used for micro-irradiation experiments, while non-irradiated cells adjacent to these fields served as controls. A continuous-wave coherent UV beam of wavelength 257 nm was focused onto a selected site of the cell nucleus with a quartz microscope objective (Zeiss Ultrafluar 32 ×/0.40 Ph), which was simultaneously used for observation of the cells in phase contrast (C. Cremer et al., 1974, 1976). Aiming was performed with the aid of a cross hair. The diameter of the microbeam at its focal site was about 1 μm, as estimated from the smallest diameter of the fluorescent spot induced on the bottom of a petri

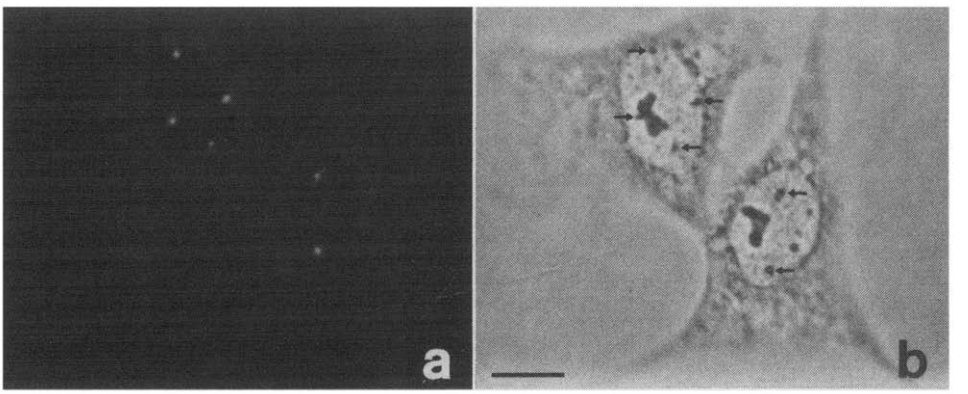

Fig. 1. Localization of micro-irradiation sites in the interphase nuclei of two M3-1 Chinese hamster cells by indirect immunofluorescence microscopy with antibodies specific for UV-irradiated DNA. The nuclei were micro-irradiated at 4 sites (left cell) and 2 sites (right cell). The irradiation time was 1/30 sec. Cells were processed for indirect immunofluorescence staining immediately after micro-irradiation (a). In phase-contrast microscopy, the sites of micro-irradiation are detectable as small dark spots (arrows). The diameter of the lesions varied between 0.85 and 1.7 μm. Bar, 10 μm.

dish (Zorn et al., 1979). Owing to the divergence of the beam above and below the focal plane and to the stray light produced when the beam passes a cellular structure, the 'effective' spot diameter in interphase nuclei fixed immediately after micro-irradiation may be somewhat larger. The immunocytochemical evidence presented in this paper (see Fig. 1) indicates an 'effective' spot diameter between 1 and 2 μm.

The UV power incident at the cell surface was estimated (C. Cremer et al., 1976) to be about 7.5×10^{-9} W. The irradiation time was controlled by a photographic shutter and varied between 1/8 and 1/30 sec.

Post-treatment of micro-irradiated cells

For M3-1 cells, 2 different protocols were used for post-treatment of microirradiated cells.

Protocol A. The cells were post-incubated (37°C) with medium containing 2 mM caffeine for 4–12 h. Colchicine (2 μg/ml) was added for the last 2 h. Then chromosome preparation was performed in situ (Zorn et al., 1976).

Protocol B. The cells were fixed immediately after micro-irradiation with acetic acid : methanol, 1 : 3 (no colchicine, no hypotonic treatment).

In some experiments, CHL cells were used instead of M3-1 cells. These cells were synchronized in G1 (Zorn et al., 1979) and micro-irradiated in this stage of the cell cycle. After micro-irradiation, the CHL cells were pulse-labelled with [^{3}H]thymidine (10 μCi/ml, 47 Ci/mmole; Amersham) for 2 h. Thereafter, the cells were washed with 1×10^{-5} M 'cold' thymidine as described (Zorn et al., 1979) and post-incubated in minimal essential medium containing 10% FCS and 0.5 mM caffeine. 30 h later, chromosome preparation was performed in situ (Zorn et al., 1976). Colchicine (1 μg/ml) was added for the last 3 h.

Autoradiography

CHL cells were processed for autoradiography as previously described (Zorn et al., 1979).

Indirect immunofluorescence staining of UV-irradiated chromatin

Details of the immunization procedure, purification and specificity of the rabbit antiserum raised against UV-irradiated DNA have been described (Cornelis and Errera, 1980). This antiserum was routinely used in the present experiments. It has a high affinity for UV-irradiated DNA and has a low affinity for non-irradiated single-stranded DNA (Cornelis, 1978).

Immunofluorescent staining of micro-irradiated chromatin was also possible with another preparation of antibodies to UV-irradiated DNA (mainly against pyrimidine dimers) (Fukuda et al., 1976; C. Cremer et al., 1980b). Sera from non-immunized rabbits and from a rabbit immunized against human haemoglobin A (gift from Dr. M. Schachner) were used as controls and yielded negative results. Cells fixed with methanol/acetic acid (3 : 1) and air-dried were rehydrated for 10 min in PBS. After removal of PBS, cells were incubated with the first antiserum (either against UV-irradiated DNA, or control) in a wet chamber for 2.5 h. Cells were then

washed for 10 min in PBS with 3 changes, incubated with fluorescein–isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1 : 30 in PBS; Nordic) for an additional 30 min at 37°C, washed again 3 times with PBS and finally rinsed with bidistilled water for 10 min. Thereafter, cells were embedded under cover glasses with Mowiol (Serva). A Zeiss photomicroscope equipped with epifluorescence illumination was used for observation and microphotographs of the cells.

Results

Indirect immunofluorescence microscopy of interphase cells and mitotic cells with partial or generalized chromosome shattering

Non-synchronized M3-1 cells were micro-irradiated at one site of the interphase nucleus and either fixed immediately or post-treated with caffeine (2 mM). 4–12 h after micro-irradiation, chromosome preparation was performed (2-h arrest with colchicine). Chromosome shattering in the M3-1 cell line induced by the synergistic effect of UV radiation and caffeine has previously been described in detail (C. Cremer et al., 1982; C. Cremer and Gray, 1982a). In UV-micro-irradiated cells,

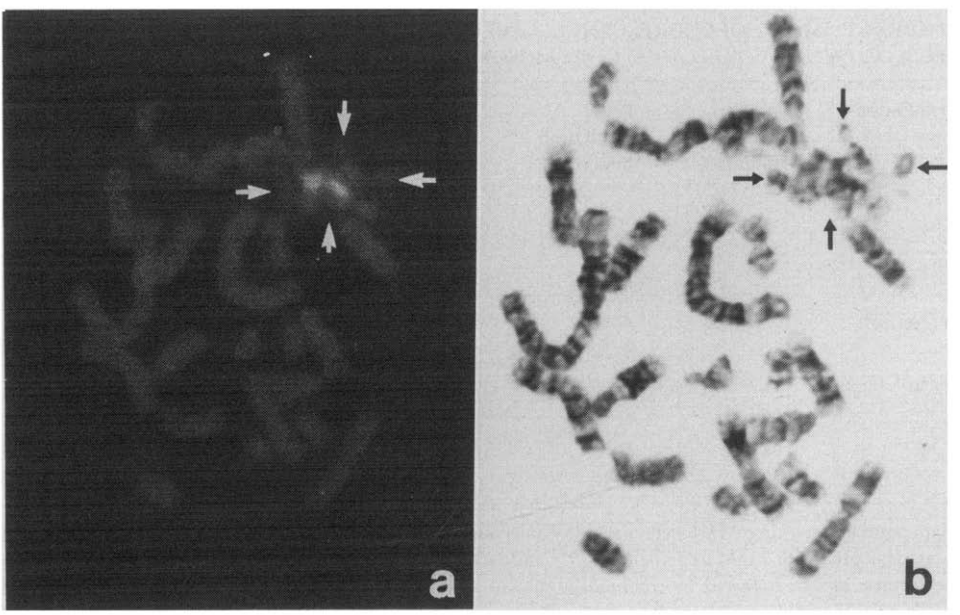

Fig. 2. Detection of pyrimidine dimers in situ by indirect immunofluorescence microscopy in a metaphase with partial chromosome shattering. The cell (M3-1) was micro-irradiated (1/30 sec) in interphase at one site of the nucleoplasm and post-treated with 2 mM caffeine for 6 h before chromosome preparation was performed. In (a) specific fluorescence can be noted within the shattered chromosome region, indicated by white arrows. Contours of intact chromosomes are visible by slight, non-specific background fluorescence. In (b) the same cell is shown after Giemsa banding. The shattered chromosome region is indicated by black arrows which correspond to white arrows in (a).

similar yields of cells with PCS and GCS were observed (data not shown) as in previous experiments with a V79 line (C. Cremer et al., 1981). After fixation the cells were processed for indirect immunofluorescence microscopy with antiserum against UV-irradiated DNA. Both micro-irradiated interphase cells and metaphase spreads obtained therefrom showed a small area of fluorescent chromatin, while non-irradiated cells stained negative. When nuclei were micro-irradiated at several sites and fixed immediately thereafter (protocol B) the localization and numbers of fluorescent spots coincided with the numbers and localization of micro-irradiated sites (Fig. 1). Interestingly, a dark lesion was noted at the micro-irradiated nuclear site after the cells had been fixed with methanol/acetic acid (3 : 1) (Fig. 1b). These lesions were not noted in phase-contrast observations of living micro-irradiated cells.

The immunofluorescent staining was well reproducible; in cells micro-irradiated in the nucleus and fixed immediately thereafter (protocol B), specific antibody-positive reactions (see Fig. 1) were clearly observed at most micro-irradiated sites ($\geq 95\%$). However, both the fraction of labelled interphase cells and the intensity of the labelling decreased after an additional post-incubation period (protocol A). This phenomenon is presently under investigation.

TABLE 1

INDIRECT IMMUNOFLUORESCENCE LABELLING IN MITOTIC CELLS WITH PARTIAL (PCS) OR GENERALIZED (GCS) CHROMOSOME SHATTERING

Irradiation time (sec)	Number of cells irradiated	Post-incubation time (h)	Total number of mitotic cells		Numbers of mitotic cells with PCS and GCS			
					Cells with PCS		Cells with GCS	
			L	NL	L	NL	L	NL
0 (control)	–	4– 7	0	203	0	0	0	0
	–	8–12	0	125	0	0	0	0
1/30	717	4– 7	2	57	2	14	0	24
	662	8–12	2	46	0	4	2	3
1/8	963	4– 7	24	86	18	13	6	35
	1302	8–12	37	71	0	0	37	38

Exponentially growing M3-1 cells were UV-micro-irradiated (irradiation times 1/30 and 1/8 sec) and post-incubated with 2 mM caffeine for 4–7 h and 8–12 h, respectively. Then chromosome preparation was performed in situ followed by immunofluorescent staining. Mitotic cells situated in the 'experimental fields' (see Material and Methods) were evaluated, except for controls (0 sec) where mitotic cells situated in fields of unirradiated cells were evaluated. For a given treatment (e.g. 1/8 sec, 4–7 h) and damage category (e.g. PCS), the number of labelled mitotic cells (L; clearly detectable immunofluorescent labelling) is given; also shown is the number of mitotic cells with no or very weak (equivocal) labelling (NL). For example, after 1/8 sec irradiation and 4–7 h incubation, a total of 24 labelled mitotic cells was observed in the 'experimental fields'. Of these 24 labelled mitotic cells, 18 were cells with PCS and 6 were cells with GCS. In all cases where a clearly detectable immunofluorescence was found, it was restricted to a small part of the metaphase plate.

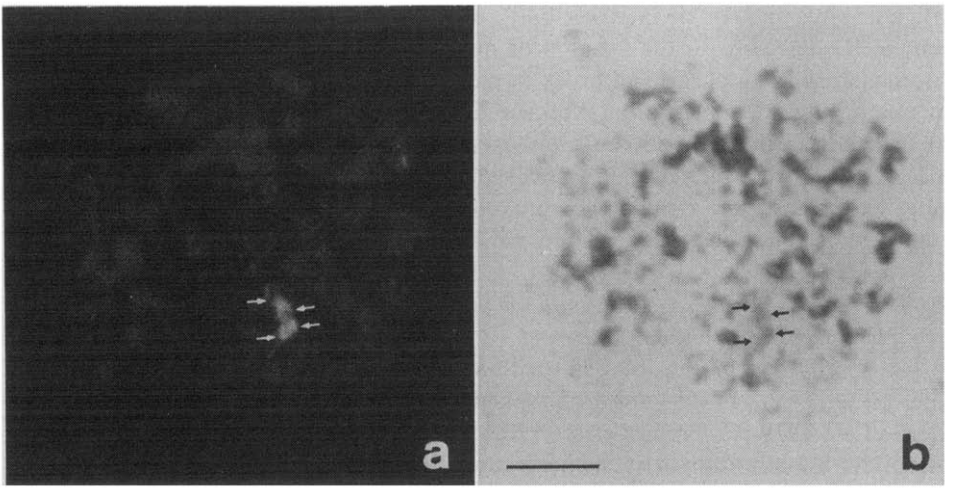

Fig. 3. Detection of pyrimidine dimers in situ by indirect immunofluorescence microscopy in an M3-1 cell with generalized chromosome shattering (a). Fig. 3b shows the same cell after Giemsa staining. Corresponding chromatin sites in (a) and (b) comprising the area of specific fluorescence are indicated by white and black arrows. The cell was micro-irradiated (1/8 sec) in interphase at one site of the nucleoplasm and post-treated with caffeine (2 mM) for 6 h before chromosome preparation was performed. Bar, 10 μm. An increase in the absolute size of the fluorescing area in mitotic cells (protocol A) as compared with the size of the fluorescing area observed in interphase cells fixed immediately after micro-irradiation (protocol B) might be due to chromatin movements during the 6-h post-incubation period and/or due to hypotonic treatment. Note, however, that the entire fluorescing area obviously comprises only a small proportion of the total area of the mitotic cell covered by chromatin. Thus, the percentage of labelled chromatin appears to be similar in interphase and in the following mitotic stage. As shown previously (C. Cremer et al., 1981), the striking difference in chromosome morphology between PCS (Fig. 2) and GCS (Fig. 3) cannot be explained by difference in UV dose, since both types of shattering may be found after identical micro-irradiation and post-incubation treatments.

In metaphase spreads with PCS, indirect immunofluorescence was found without exception to be localized in the area with shattered chromatin (Fig. 2; for frequency see Table 1). Notably, the size of this area was sometimes larger than the labelled region. In cells with GCS, indirect immunofluorescence was always restricted to a small part of the chromatin (Fig. 3; Table 1). The distribution of DNA photolesions as shown by the fluorescent region, however, does not fit the generalized pattern of chromosome shattering.

We have previously shown (C. Cremer et al., 1981), that PCS and GCS can be obtained at the first post-irradiation mitosis in cells micro-irradiated in G1 and S, respectively, and post-treated with caffeine, but not in cells micro-irradiated in G2. The time protocol used in the present experiments suggests that most of the cells with shattered chromosomes were in S phase at the time of micro-irradiation.

Although PCS and GCS were obtained by the synergistic effect of UV-micro-irradiation and caffeine post-treatment, only a fraction of these cells showed indirect immunofluorescence labelling (Table 1). In this unlabelled fraction, the number of

antigenic sites and/or their accessibility to the antibodies obviously was not sufficient to be detected. In the fraction of mitotic cells that showed apparently intact chromosomes or an occasional break or gap, label was absent or appeared too weak for unequivocal demonstration. Absence of label from these cells was partly due to the fact that, in these experiments, only some 85% of the total number of interphase nuclei in the 'experimental fields' received micro-irradiation. Furthermore, it is well conceivable that there may be a reduction in antibody binding to chromosomes of irradiated cells not displaying PCS or GCS.

Distribution of micro-irradiation-induced unscheduled DNA synthesis (UDS) in cells with generalized chromosome shattering

The spatial distribution of UDS within micro-irradiated nuclei as well as UDS-label patterns on chromosomes of the subsequent metaphase have been described (Zorn et al., 1979; C. Cremer et al., 1981; T. Cremer et al., 1980, 1982). In contrast to indirect immunofluorescence microscopy, the UDS-labelling method can only be used in cells outside S phase. On the other hand, this method is suited for the following of micro-irradiated chromatin from interphase to metaphase when there is

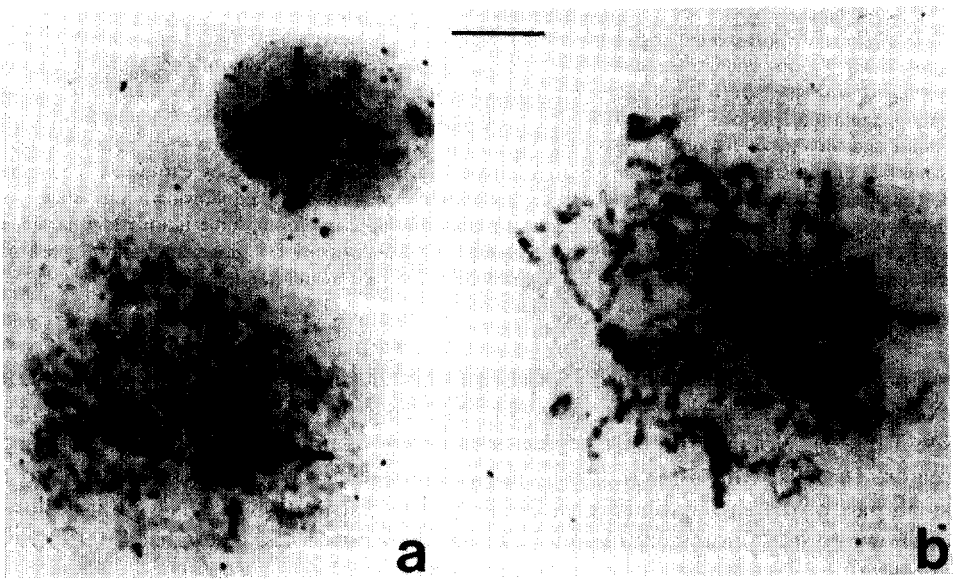

Fig. 4. Autoradiographs of three CHL cells after UV-microirradiation (1/15 sec) of the cells in G1 at one site of the nucleoplasm. Cells were pulse-labelled with [^{3}H]thymidine (10 μCi/ml; spec. act. 47 Ci/mmole) for 2 h immediately after micro-irradiation and thereafter allowed to proceed through the cell cycle for an additional 30 h in the presence of caffeine (0.5 mM) before chromosome preparation was performed. Arrows indicate sites of unscheduled DNA synthesis in the nucleus of an interphase cell (a, upper part) and in 2 other cells with generalized chromosome shattering (a, lower part, and b). Specific labelling was restricted to a small part of the chromatin in all cells. In (a) the shattered chromosome complement appears 'pulverized', while in (b) the structure of chromosomes exhibiting an apparent failure of condensation into normal metaphase chromosomes can still be recognized. Bar, 10 μm.

a great reduction of the number of DNA photolesions owing to excision repair. Here, CHL cells were synchronized in G1 by mitotic selection and serum starvation (0.5% FCS). After partial UV-irradiation of the nucleus, cells were pulse labelled with [^{3}H]thymidine for 2 h and further incubated with 0.5 mM caffeine under normal growth conditions (10% foetal calf serum) for 30 h. During the last 3 h of post-incubation, colchicine was added and chromosome preparation was followed by autoradiography. In total, 12 UDS-labelled mitotic cells (the number of grains per cell was clearly above background) with GCS were found in the experimental field; no cells with PCS were obtained. In control cells, chromosome shattering was not observed.

In all 12 UDS-labelled mitotic cells exhibiting GCS, UDS label was restricted to a small part of the chromosome complement (Fig. 4). The silver-grain density outside the labelled area did not exceed background levels counted over non-irradiated cells of the same dishes. Silver grains also accumulated at one site over micro-irradiated interphase nuclei (Fig. 4a). A label pattern as shown in Fig. 4 was never observed over cells with GCS obtained after whole-cell irradiation.

Discussion

Partial and generalized chromosome shattering (PCS and GCS) can be obtained by the synergistic effect of UV-micro-irradiation of the interphase nucleus and caffeine post-treatment in Chinese hamster cells (Zorn et al., 1976; T. Cremer et al., 1980, 1981; C. Cremer et al., 1981). The present experiments show that indirect immunofluorescence labelling with antibodies against UV-irradiated DNA (1) coincides with the site(s) of micro-irradiation in the interphase nucleus and (2) is restricted to a small part of the chromosome complement both in cells with GCS and with PCS. The antibodies used in this study recognize predominantly, if not exclusively, pyrimidine dimers in a DNA structure (Cornelis et al., 1977; Cornelis, 1978).

In the present investigation, concentration of DNA photolesions to a small part of the chromosome complement in micro-irradiated cells exhibiting GCS was further observed by an independent approach, with unscheduled DNA synthesis (Fig. 4).

Although serum starvation is an effective means of G1 arrest of CHL cells, a small proportion of the cells was still able to enter S phase (Zorn et al., 1979). Furthermore, because in rare cases ($< 1\%$ of mitotic cells) GCS was observed without UV-irradiation (C. Cremer et al., 1981; Cremer and Gray, 1982a), one could argue that some of the labelling may have been due to semi-conservative DNA synthesis. For example, it might be assumed that such a localized labelling represents late-replicating chromatin. However, it has been found (Taylor, 1960; Hsu, 1964; Deaven and Petersen, 1973; Cremer and Gray, 1982b) that, besides the sex chromosomes, chromosomes 10,11 contain large amounts of late-replicating material. Furthermore, in the CHL cells used here, no strong spatial association of these chromosomes was found (L. Hens et al., in preparation). Thus, it is regarded as highly unlikely that the type of localized labelling observed here is due to semicon-

servative DNA synthesis in late S phase. By using the autoradiographic data by Stubblefield and Gay (1970), similar arguments can be made against the hypothesis that the labelling might be due to synthesis in some other part of S phase.

We conclude that the localized labelling obtained after UV-micro-irradiation of the nucleus and post-treatment with caffeine (Fig. 4) is indeed due to unscheduled DNA synthesis and not to semiconservative DNA synthesis. This conclusion is consistent with the observation that such a localized labelling was never found over cells with GCS obtained without UV-irradiation.

In cells with GCS the distribution of DNA photolesions as shown by immunofluorescence and UDS labelling obviously does not fit the generalized pattern of chromosome shattering. Evidence for a territorial organization of chromosomes in the interphase nucleus of Chinese hamster cells has been reported (Stack et al., 1977; Zorn et al., 1979; T. Cremer et al., 1982). Accordingly, the UV microbeam hits only a few chromosomes or chromosomal segments when focused onto the interphase nucleus. We conclude that chromosomes that were not micro-irradiated participated in the process of chromosome shattering in GCS. Additional evidence that stray UV radiation per se does not account for this effect has been described elsewhere (C. Cremer et al., 1981; T. Cremer et al., 1981).

In cells with PCS, strong immunofluorescence was observed in the shattered area, whereas the intact chromosomes were not labelled. The occurrence of shattered, but non-labelled, chromatin clustered around the labelled region can possibly be attributed to stray radiation, which might produce additional DNA photolesions close to the micro-irradiated site. The number of these lesions might be sufficient to induce shattering but might not be sufficient to be detected by indirect immunofluorescence microscopy.

Our microbeam experiments show for the first time the distribution of DNA photolesions as compared with the distribution of shattered chromosomes in single cells. They provide further evidence for the idea that 2 levels of chromosomal damage exist in Chinese hamster cells after UV micro-irradiation of the interphase nucleus and caffeine post-treatment: level 1 is restricted to micro-irradiated chromosomes, while level 2 involves both irradiated and non-irradiated chromosomes (C. Cremer et al., 1981).

Caffeine inhibits daughter-strand repair (Lehmann, 1976). The synergistic effect of this compound on the yield of chromosome aberrations in UV-irradiated cells has been ascribed to this inhibitory effect, although the relationship between the effects observed at the molecular and the cytogenetic levels is still far from clear (Nilsson and Lehmann, 1975; Kihlmann, 1977). So far, it seems reasonable to assume that chromosomal aberrations are induced exclusively in UV-irradiated DNA strands. If the hypothesis be made that in a similar way chromosome shattering induced by UV radiation and caffeine is restricted exclusively to UV-irradiated chromatin, such a hypothesis would be consistent with level-1 damage as exemplified by PCS but does not provide a ready explanation for level-2 damage as seen in GCS. Instead, our data suggest that a more indirect mechanism accounts for GCS. A detailed discussion of the possible mechanisms of such an indirect effect has been published elsewhere (T. Cremer et al., 1980, 1981; C. Cremer et al., 1981).

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