RADIATION RESEARCH 66, 106–121 (1976)

# Effects of Laser UV-Microirradiation ($\lambda = 2573$ Å) on Proliferation of Chinese Hamster Cells[1]

CHRISTOPH CREMER,[2] THOMAS CREMER, CHRISTIAN ZORN, AND LILI SCHOELLER[3]

*Institut für Humangenetik und Anthropologie der Universität Freiburg i. Br., D-7800 Freiburg/Br., Albertstr. 11*

CREMER, C., CREMER, T., ZORN, C., AND SCHOELLER, L. Effects of Laser UV-Microirradiation ($\lambda = 2573$ Å) on Proliferation of Chinese Hamster Cells. *Radiat. Res.* 66, 106–121 (1976).

A laser uv-microbeam with a wavelength of 2573 Å having a minimum spot diameter of approx 0.5 μm was used to microirradiate interphase cells of a V-79 subline of Chinese hamster cells. The incident energy necessary to induce a significant decrease of proliferation was 30 to 60 times larger after microirradiation of cytoplasm as compared with microirradiation of nucleoplasm. The mean value of relative cell numbers 40 hr after irradiation as a function of incident energy did not differ whether the cells were microirradiated lying singly or together in small groups. Analysis of individual growth curves of singly lying cells microirradiated in the nucleoplasm with the same energy showed heterogeneous reactions. The incident energy per cell compatible with proliferation of about 50% of the cells after microirradiation of nucleoplasm was approx. $2 \times 10^{-3}$ ergs. From this value it is suggested that the energy density within the focus was in the region of several thousand ergs per square millimeter. Photochemical effects are thought to be the cause of growth disturbance,while thermal effects are excluded.

## INTRODUCTION

By application of a laser uv-microbeam, lesions with a diameter of approx 0.5 μm, as seen by light microscopy, can be produced within living mammalian cells (*1*). Compared to monochromatic uv-microbeams of similar wavelength using incoherent ("conventional") light sources (*2*), the maximum intensity of the beam at the focal point is larger by several orders of magnitude. The much higher intensities combined with a smaller spot size possibly induce damage in an irradiated cell which differs in some respect from the kind of damage known from work with conventional uv-microbeams (*3*). For example, one has to consider whether the high focal intensity attainable by a laser uv-microbeam could give rise to thermal denaturation, in contrast to isothermic effects produced by conventional uv-micro-

[1] This investigation was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 46).

[2] Parts of this investigation will be presented in the doctoral dissertation of C. Cremer to be submitted to the faculty of Biology, University of Freiburg i. Br.

[3] Zentrallaboratorium für Mutagenitätsprüfung, D-7800 Freiburg i.Br., Breisacherstr. 33.

irradiation with comparable energies. With respect to the nucleus it seems possible that the accumulation of photoproducts in a small part of the chromatin induced by laser uv-microirradiation within a few milliseconds gives rise to effects not observed when conventional uv-microbeams are used. In the latter, as a rule, the spot size is larger and the irradiation time considerably longer (*2*).

This paper reports on effects on the proliferation of cells after laser uv-microirradiation of cytoplasm or nucleoplasm of a V-79 subline of Chinese hamster cells. Two questions were of major interest:

(a) How does laser uv-microirradiation of nucleoplasm or cytoplasm influence cell growth?

The results are compared with the effects reported by other authors who performed partial irradiation of cells with conventional uv-microbeams or irradiation of whole cells.

(b) Does the distance between cells influence the effects of microirradiation on cell growth?

The distance between microirradiated cells may be important for proliferation. For example, neighboring cells might compensate defects by metabolic cooperation, or damaged cells might release substances to the medium which also impair the growth of neighboring cells. As far as we know, former reports concerning the proliferation of mammalian cells after uv-microirradiation refer to cells lying together in small groups (*4*), while the proliferation of singly lying cells after uv-microirradiation has not been investigated. Therefore, in the investigation presented here we performed laser uv-microirradiation experiments on cells lying either singly or together in groups of four cells each.

## MATERIALS AND METHODS

The laser uv-microbeam of wavelength 2573 Å has been described in detail elsewhere (*1*, *5*). A continuous-wave argon-ion laser beam with a wavelength of 5145 Å is transmitted through an ammonium dihydrogenphosphate (ADP) crystal. By this means, frequency doubling occurs and a coherent continuous wave ultraviolet beam with a wavelength of 2573 Å is emitted. The uv-beam, after having passed a photographic shutter to control the duration of irradiation ($t_{irr}$), enters the irradiation microscope. There, the optical arrangement is similar to the one used in a fluorescence incident light microscope. The uv-beam, having passed an adapting lens, is reflected downwards with the aid of a selective uv-mirror in such a way that the beam becomes coaxial with the optical axis of the microscope. The beam then enters a quartz objective (Zeiss Ultrafluar 100/0.85 Ph Glyz), simultaneously used for irradiation and phase-contrast observation. The specimen to be irradiated lies below a quartz coverglass. Optical contact between objective and coverglass is obtained with glycerine (Fig. 1).

With the use of the full aperture of the objective (half-aperture angle 39°), the uv-beam is focused so that a spot with a minimum diameter of approx 0.5 $\mu$m is produced. This diameter was determined with different light-microscopic procedures (fluorescence, observed in the specimen plane; lesions in stained cell preparations and in unstained living cells) (*1*).

Before entering the photographic shutter, the power of the uv-beam was continually monitored with the aid of an uv-detector system to which a small fraction $P_D$ of the uv-beam was diverted. The uv-power $P_{ap}$ entering the aperture stop of the objective was measured before and after each irradiation experiment by means of a photodiode (PIN 10 UV, UDT, Santa Monica, Calif.) that was inserted instead of the objective. Between two measurements of $P_{ap}$, $P_D$ was kept constant by regulating the laser current. Measurements of $P_{ap}$ at 1-hr intervals indicated a maximum variation of $\pm 10\%$. The energy $E_{ap}$ entering the objective during an irradiation event was calculated with $E_{ap} = P_{ap} \times t_{irr}$ and is given in relative units, FE. The error of $E_{ap}$ was estimated to be maximum $\pm 15\%$. To obtain $E_{ap}$ in ergs, the photovoltage of the photodiode mentioned above was calibrated with a second photodiode (PIN CAL UV, UDT, Santa Monica, Calif.) which had been calibrated by the manufacturer for 2500, 3200, and 3600 Å, including the range of intensities used in our experiments. From these data, it was calculated that $E_{ap} = 1$ FE corresponds to an energy of $1.1 \pm 0.2$ ergs delivered to the objective. To obtain an estimation of the energy delivered to an irradiated cell, $E_{inc}$, measurements of the beam power in front of and behind the objective, of the transmission of the quartz cover glass, of the glycerine immersion fluid, and of the culture medium which contained 15% fetal calf serum were made. The transmission measurements indicate that the energy losses between the entrance aperture of the objective and the cell level are mainly due to losses caused by the objective, the "transmission" of which was found to be approx 20%. Compared with this, losses between the front lens of the objective and the cell level were small (transmission $\geq 85\%$), despite the high extinction coefficient of fetal calf serum. This is due to the thinness of the layer of medium, being $\lesssim 120$ μm. The optical arrangement was kept constant in all experiments. The small variations in the distance between cover glass and cell which occur in different experiments should therefore have no appreciable influence on the total transmission between entrance aperture and cell level (approx 17%). From these data, it was calculated that $E_{ap} = 1$ FE corresponds to $E_{inc} \approx 0.2$ ergs. In the following, an irradiation energy given in ergs refers to $E_{inc}$, while FE refers to $E_{ap}$. The absolute energies given here are somewhat lower than those given in a preliminary report (*5*). This we think is due to the improved methods in calibration and control of the uv-energy described in this paper.

The cell material was derived from a clone which had been established in 1972 from a Chinese hamster cell line [V-79-122 D1 (*6*)]. These cells have large nuclei with clearly visible nucleoli and a high plating efficiency (>90%). The doubling time was found to be approx 16 hr, irrespective of cell density; only at very high cell densities was cell growth impaired. Cells were grown in Eagle's MEM supplemented with nonessential amino acids and 15% fetal calf serum in a humidified atmosphere with 5% $CO_2$ at 37°C. From cells in logarithmic growth phase, a single-cell suspension was established. Two hundred to 500 cells per dish were added to each plastic petri dish (Nunc/Denmark, $\phi$ 5 cm) and incubated for 4 hr. Singly lying flattened interphase cells of clear morphology were selected for irradiation. The minimum distance to neighboring cells was 500 μm. The position of a selected cell in the petri dish was determined with the aid of a system of coordin-

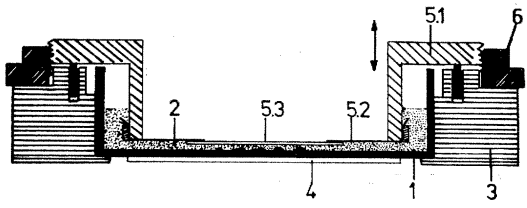


FIG. 1. The irradiation chamber: Modification of a chamber developed by P. Hösli (cross section in schematic representation). A petri dish (1) containing cells and feeding fluid (2), is inserted into the bottom part (3) of the chamber. A "cell finder" (4) (see text) is attached to the bottom of the dish. The upper part of the chamber (5.1–5.3) is adjustable in height by turning the level adjustment ring (6). A special guide prevents rotation of the metal holder (5.1) during its up-and-down movements. Into the metal holder (5.1) a thin plastic foil (5.2) is tightly clamped. In the center of the foil, a space (28 × 21 mm) is cut out and a quartz coverglass (5.3) 30 × 23 × 0.35 mm) is glued in. Observation and irradiation are done with a phase contrast objective (Zeiss Ultrafluar 100/0.85 Glyz Ph) through this quartz cover glass. The optical contact between cover glass and objective is achieved with glycerine immersion fluid. Compared to the Hösli chamber, the alterations are: (a) a modification permitting insertion of a petri dish, (b) the pasting in of a cover glass in the midst of the foil.

ates placed on a glass disc attached to the outside bottom of the pertri dish ("Cell Finder," Microlab/Arnhem/The Netherlands). For irradiation a slightly modified culture tissue chamber (Tecnomara, Zürich/Switzerland), into which the petri dish was inserted (Fig. 1), was used.

Five to 10 hr after the cells were added to the petri dishes, cells were irradiated either in the cytoplasm or in the nucleoplasm. In the first case, the distance of the irradiation site from the edge of the nucleus was approx 3 $\mu$m. In the second case, a site in the central part of the nucleus was irradiated. Nucleoli were excluded as a target. All irradiations were carried out at 22°C ambient temperature. In one petri dish, up to 10 cells were irradiated with different uv-energies. One hour was usually required to localize and irradiate these cells. Neighboring cells, also lying under the quartz cover glass, served as controls. Their growth did not differ, whether these cells were focused with the ultrafluar objective (sham-irradiated) or not. After irradiation, the petri dishes, containing irradiated cells and control cells, were kept in the incubator at 37°C. The proliferation of the individual cells was observed by counting the cells of each clone using an inverted phase contrast microscope (10×, or 32× objective). The first count was made immediately after removing the petri dish from the irradiation chamber. (Cells which had been selected but which were not relocalized after irradiation were excluded from further consideration.) The intervals between two counts were 10 to 30 hr. In most cases, counting was finished approx 80 hr after irradiation, this time corresponding to five generations of the control cells. In addition to singly lying cells, cells in small clones, each containing four cells whose distances from each other were smaller than three cell diameters, were irradiated. Cells were added to petri dishes as described above and incubated for 25 to 30 hr. Clones were selected in which all four cells had nuclei with clear morphology and were flattened. All cells of a clone were irradiated with the same energy and duration of irradiation, either in the cytoplasm or in the nucleoplasm as described for singly lying cells. Neighboring

clones of equal size served as controls. After irradiation, the clones were incubated at 37°C. Cells were either counted at intervals of 10 to 15 hr, or they were fixed 40 hr after irradiation, stained and counted.

## RESULTS

### *Microirradiation of Singly Lying Cells*

A survey of the number of cells and type of irradiation is given in Table I. Eighty cells were irradiated in the nucleoplasm and 44 cells in the cytoplasm. Individual growth curves of these cells and of 350 control cells were established.

In Fig. 2 the growth curves of cells irradiated with the same energy are summarized to give average growth curves $N(t)$. Whereas $N(t)$ following irradiation of cytoplasm is not changed or is only slightly changed compared to controls (Fig. 2b), an increasing energy $E_{ap}$ results in an increasing retardation of growth following irradiation of nucleoplasm (Fig. 2a). At 0.08 FE, no division was observed and cells were lost from the substratum. Figure 3 shows $N(t)$ 40 hr after irradiation, as a function of $E_{ap}$. Already at 0.02 FE the $N(40\text{ hr})$ value for cells irradiated in the nucleoplasm lies below the range of the controls and continues to decrease with increasing energies. In contrast, for cells irradiated in the cytoplasm, the $N(40\text{ hr})$ values are within the range of the controls for the whole energy range tested (0.02 to 0.6 FE). An analysis of individual growth curves (examples given in Fig. 4) shows that the retardation of $N(t)$, following irradiation of nucleoplasm, results from a superposition of heterogeneous responses of the irradiated individual cells (Tables II and III). The criteria in Table II are (a) the ability of the irradiated cells to divide once or more often, as characterized by the maximum cell number of a clone obtained within 80 hr after irradiation, and (b) the occurrence of abortive colonies; i.e., all cells died within the observation time of 80 hr after having divided once or several times. With increasing energy, there is a marked increase in the proportion of nondividing cells (cell number = 1). Most

TABLE I

Synopsis of Irradiation Experiments Performed with Singly Lying Cells

| *Irradiation site* | $t_{irr}$ (*sec*) | $P_{ap}$ (*FE/sec*) | *UV-energy* (*FE*) | *Number of cells* |
|---|---|---|---|---|
| Nucleoplasm | 1/125 | 0.4 to 0.9 | 0.003 to 0.007 | 12 |
| | 1/30 | 0.3 to 0.6 | 0.01 to 0.02 | 49 |
| | 1/30<br>1/15 | 0.9 to 1.2 | 0.04 to 0.08 | 19 |
| Cytoplasm | 1/30<br>1/15 | 0.6 to 1.2 | 0.02 to 0.08 | 14 |
| | 1/4<br>1/2 | 0.8 to 1.2 | 0.2 to 0.6 | 29[a] |
| | 1 | 1.2 | 1.2 | 1 |
| Controls | — | — | — | 350 |

[a] Ten cells were observed only until $t$ = 40 hr after irradiation.

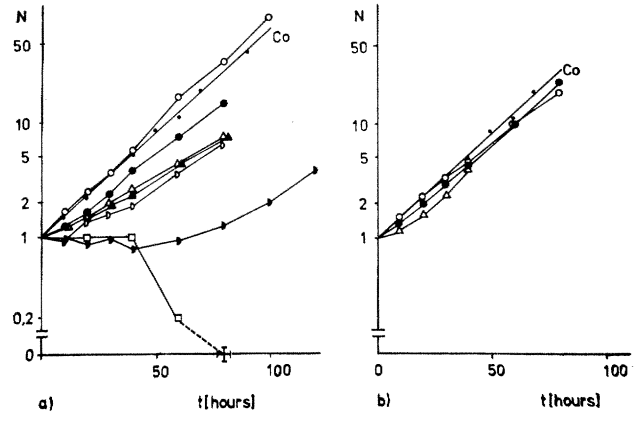


FIG. 2. Average growth curves of singly lying cells after laser uv-microirradiation. The average cell number, $N$, is plotted as a function of time $t$ (hours) after irradiation. To obtain $N(t)$, the cell numbers $N_s(t)$ of the individual growth curves (e.g., Fig. 4) were determined for $t$ = 10 hr, 20 hr, . . . , if necessary, by interpolation. Co, average growth of unirradiated controls (sample of 30 cells); ✠, all cells have lysed or detached from the substratum at the time indicated by the cross. (a) Average growth following irradiation of nucleoplasm: ○, 0.003 FE (6 cells); ●, 0.007 FE (6 cells); △, 0.01 FE (26 cells); ▲, 0.02 FE (23 cells); ▯, 0.04 to 0.05 FE (6 cells); ◗, 0.06 FE (8 cells); □, 0.08 FE (5 cells). (b) Average growth following irradiation of cytoplasm: ○, 0.02–0.08 FE (14 cells); ●, 0.2–0.3 FE (11 cells); △, 0.6 FE (14 cells).

of these cells (approx 80%) lysed within 80 hr and/or detached from the substratum. The proportion of cells having divided only once after being irradiated (cell number = 2) is relatively small, as was the proportion of cells which had proliferated only to 3 or 4 cells within the observation time. The proportion of clones which reached a size of 5 or 16 cells (Table II) within 80 hr, is large for controls and at least for cells irradiated in the cytoplasm, but diminishes quickly with increasing energy for cells irradiated in the nucleoplasm. Those cells which were able to divide once after irradiation in most cases showed further proliferation

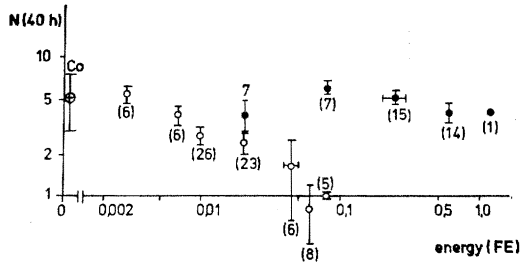


FIG. 3. Laser uv-microirradiation of singly lying cells. Average cell number 40 hr after irradiation, $N$(40 hr), as a function of the energy delivered per cell (FE) following irradiation of nucleoplasm (○) and cytoplasm (●). (Double logarithmic plot) Co: mean value of controls with standard deviation, SD, calculated by evaluating a sample of 170 individual growth curves. For irradiated cells, the vertical bars indicate the deviations of the mean values, SDM. In two cases, experiments within the energy range indicated by horizontal bars have been combined. The numbers of cells irradiated with the same energy are given in parentheses.

TABLE II

Ability of Singly Lying Cells to Divide after Laser-UV-Microirradiation[a]

| *Irradiation site* *UV-energy (FE)* | | *Controls* | *Nucleoplasm* | | | *Cytoplasm* | | |
|---|---|---|---|---|---|---|---|---|
| | | *0* | *0.003–0.007* | *0.01–0.02* | *0.04–0.08* | *0.02–0.08* | *0.2–0.3* | *0.6* |
| Number of experiments ($n_0$) | | 350 | 12 | 49 | 19 | 14 | 11 | 8 |
| (a) Maximal cell number within 80 hr after irradiation | $1n$ | 4 | 0 | 17 (r) | 14 (r) | 0 | 1 (r) | 2 (r) |
| | $2n$ | 13 | 1 | 3 | 0 | 2 (r) | 1 | 1 (r) |
| | $3,4n$ | 18 | 0 | 5 | 3 (r) | 0 | 0 | 0 |
| | $\geq 5n$ | 315 | 11 | 24 (d) | 2 | 12 | 9 | 5 (d) |
| | $\geq 16n$ | 245 | 9 | 5 (d) | 1 (d) | 10 | 5 (d) | 3 (d) |
| (b) Abortive colonies, with maximal cell number | $2n$ | 13 | 1 | 3 | 0 | 0 | 0 | 0 |
| | $3n$ | 1 | 0 | 2 (r) | 1 (r) | 0 | 0 | 0 |
| | $4n$ | 3 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] $n_0$, number of cells irradiated; $n$, number of cases which met the criterion stated at left. For each figure a "confidence range" (CR) of the proportion $n/n_0$ was calculated under the assumption of a binomial distribution. If CR-values for irradiated cells of a given category did *not* overlap with the corresponding CR-values for controls, a significant rise (r) or decrease (d) was assumed.

TABLE III

Division Delay of Singly Lying Cells after Laser-UV-Microirradiation[a]

| *Irradiation site* / *UV-energy (FE)* | *Controls* | *Nucleoplasm* | | | *Cytoplasm* | | |
|---|---|---|---|---|---|---|---|
| | *0* | *0.003–0.007* | *0.01–0.02* | *0.04–0.08* | *0.02–0.08* | *0.2–0.3* | *0.6* |
| Number of experiments with at least one division ($n_1$) | 346 | 12 | 32 | 5 | 14 | 10 | 6 |
| Division delay 10–30 hr ($n$) | 9 | 5 (r) | 8 (r) | 0 | 0 | 1 (r) | 1 (r) |
| Division delay, 30 hr | 2 | 0 | 1 | 2 (r) | 0 | 0 | 0 |

[a] $n_1$, number of cells which divided after irradiation. For total number of irradiated cells ($n_0$), see Table II. $n$, number of cells showing a division delay. For each figure a "confidence range" (CR) of the proportion $n/n_1$ was calculated under the assumption of a binomial distribution. If CR-values for irradiated cells of a given category were higher than the corresponding CR-values for controls, a significant rise (r) was assumed.

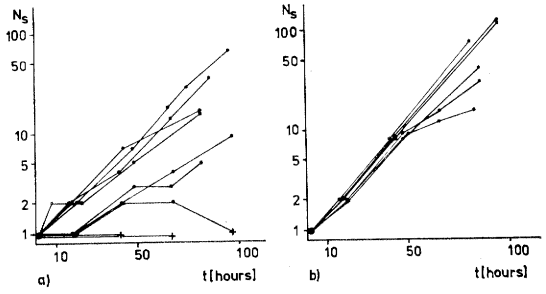


FIG. 4. Examples of individual growth curves of singly lying cells after laser uv-microirradiation (semilogarithmic plot). $N_s$, cell number of a clone; $t$, time after irradiation; ✠, cell lysed. (a) Proliferation after irradiation of nucleoplasm with $E_{ap}$ = 0.01 FE. The graph shows a sample of nine individual growth curves of the total of 26 cells irradiated with this energy. (b) Proliferation after irradiation of cytoplasm with $E_{ap}$ = 0.3 FE. The growth curves of all six cells irradiated with this energy are shown.

so that a clone size of at least 5 cells was reached within 80 hr. From this clone size it is concluded that, following the first division after irradiation, at least two subsequent cell cycles have been completed.

As Table IIb indicates, abortive clones occurred very rarely within the time investigated. Division delay (Table III) was observed in a considerably higher proportion of cells which divided after irradiation of the nucleoplasm compared to control cells. The delay possibly increases with higher irradiation energy. Development of giant or polynucleate cells, described as occurring frequently in mouse-L-cells after uv-microirradiation (*4*), was a rare event both in control cells and in irradiated cells.

*Microirradiation of Small Clones Each Containing Four Cells*

The cells of 14 clones were irradiated in the cytoplasm, the cells of 49 clones were irradiated in the nucleoplasm, and 90 unirradiated clones served as controls.

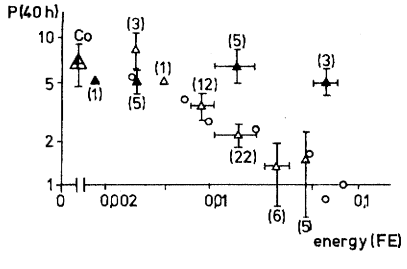


FIG. 5. Relative cell number 40 hr after irradiation, $P$(40 hr): = $N$(40 hr)/$N$(0 hr), of small clones each containing four cells. $P$(40 hr) is given as a function of the energy applied per cell (FE). The plot is double logarithmic. For notation see Fig. 3. Co, $P$(40 hr) from 90 control clones; △, irradiation of nucleoplasm (49 clones); ▲, irradiation of cytoplasm (14 clones); ○, $P$(40 hr) values for singly lying cells, after iradiation of nucleoplasm, from Fig. 3. For controls, the standard deviation SD, calculated by evaluating a sample of 90 individual growth curves, is given by vertical bars. For irradiated cells, the vertical bars indicate the deviations of the mean values, SDM. In five cases, experiments within the energy range indicated by horizontal bars have been combined. The numbers of cells irradiated with the same energy (range) are given in parentheses.

Figure 5 shows the relative cell number 40 hr after irradiation [$P$(40 hr)]. Whereas the $P$(40 hr) values of cells irradiated in the cytoplasm showed no difference compared to the control groups in the energy range tested (0.003 to 0.06 FE), the $P$(40 hr) values of cells irradiated in the nucleoplasm quickly diminished in the same energy range. At 0.06 FE, almost no increase of $P$(40 hr) was observed. For comparison, the corresponding values for single cells from Fig. 3 are plotted. Considering the degree of accuracy given in these experiments, no difference is seen in $P$(40 hr) whether the cells were irradiated lying singly or together in small clones.

## DISCUSSION

The main points of the following discussion are:

(a) spatial extent of nuclear lesions produced by laser uv-microirradiation;
(b) influence of distance between microirradiated cells;
(c) comparison of the sensitivity of cells microirradiated either in the nucleoplasm or in the cytoplasm with the same energy;
(d) heterogeneity of cell growth in different cells after the same microirradiation treatment, and possible causes which might contribute to this phenomenon;
(e) comparison of energies and energy densities used for laser uv-microirradiation of cells with data known from uv-microirradiation with a conventional uv-source and from uv-irradiation of cells as a whole;
(f) theoretical considerations concerning the possibility of thermal effects by laser uv-microirradiation.

(a) After laser uv-microirradiation of either nucleoplasm or cytoplasm with energies and intensities used for the experiments presented in this paper, we saw no alteration of the irradiated cell site by light microscopic observation. However, using much higher energies and intensities, dark spots appeared in the nuclei of unstained living cells, the "lesions" having a diameter of approx 0.5 $\mu$m (*1*). Lesion diameters, as seen by light microscopic observation, were found to be significantly smaller than the largest diameter of the cone of rays within the nucleus, calculated from the aperture angle. They were denoted as "effective" spot diameters. To study the correlation between spot size and spatial extent of DNA damage induced by laser uv-microirradiation, human fibroblasts were irradiated in the nucleoplasm and labled with a pulse of $^3$H-thymidine. Autoradiographic findings indicated that a local repair synthesis (*7*, *8*) occurred to eliminate DNA lesions induced by laser uv-microirradiation (Zorn, Cremer, and Cremer, unpublished results). The area of local repair was small in comparison with the nuclear area; however, its diameter was found to be larger than the "effective" spot diameter defined above. This observation suggests that DNA photoproducts are produced in the whole cone of rays within the nucleus.

(b) As Fig. 5 shows, cell growth was very similar, regardless of whether the microirradiated cells lay singly or together in clones of four cells each. So far, neither positive nor negative interactions of microirradiated cells with regard to proliferation were detected. But when all cells of small clones, except one or two, were microirradiated with high energies ($\gg$1 FE) leading to cell death within a few hours, it was observed that the unirradiated cells sometimes died also (unpub-

lished results). For technical reasons, we have been unable, as yet, to decide whether the proliferation of a single microirradiated cell could be improved by unirradiated adjacent cells.

(c) Figures 2–5, and Tables II and III show that there is a large difference between the proliferation of cells irradiated in the nucleoplasm and cells irradiated in the cytoplasm. According to Table II, the incident energy, at which a significant increase of the proportion of nondividing cells was first observed following irradiation of nucleoplasm, fell in the range from 0.01 to 0.02 FE. The incident energy required to produce this effect with cytoplasmic irradiation, was approx 30 to 60 times larger.

A similar figure is obtained if one compares the proportion of cells which proliferated to clones with at least five cells within 80 hr after irradiation. A large difference of sensitivities is also suggested if division delay is considered (Table III).

If one assumes the uv-absorbance by nucleoplasm and cytoplasm to be 50 and 30%, respectively (*9*), the sensitivity difference resulting for the energies absorbed is still considerably larger than 10.

Smith and Dendy also found significant differences in the effects on proliferation of mouse L-cells between microirradiation in the nucleoplasm ("nuclear sap") and in the cytoplasm (*4*). Their experiments were performed on colonies of four to eight cells using an incoherent heterochromatic uv-source and a spot diameter of 3.5 $\mu$m. In contrast to our results, however, irradiation of the cytoplasm required an incident energy only two times larger than irradiation of the nucleoplasm to disturb cell growth clearly. The question, remains, however, whether this difference from our results is mainly due to differences of the microbeams regarding wavelength, irradiation intensity, spot diameter, or due to differences in cell material, culture conditions, and ambient temperature during irradiation.

(d) Individual growth curves (Fig. 4a) show that the retardation of average growth (Fig. 2a) after microirradiation of nucleoplasm is due to heterogeneous responses of the single cells: Microirradiation with the same energy, duration of irradiation, and spot size either led to cell death, or induced only a division delay, or had no detectable influence on cell proliferation at all (Tables II and III). A wide variation in effects produced by the same microirradiation treatment was also found by Smith and Dendy (*4*). A large variety of causes may contribute to this heterogeneity, a phenomenon which is, of course, well known from other kinds of irradiation experiments (*10*). Therefore, the reasons mentioned here, with regard to the conditions of our microbeam experiments, are only some of many possible aspects.

1. Since the cells were not synchronized before irradiation, differences in position of the irradiated cells in the cell cycle must be taken into consideration (*11*).

2. A genetic heterogeneity of the cell line used might lead to differences in uv-sensitivity of cells (*12*). The influence of parameters 1 and 2 on heterogeneity can be controlled much better as soon as synchronized, euploid cells of high colony-forming ability become available to us.

3. Two points have to be considered with regard to a contribution of chromatin damage to the different fates of irradiated cells. First, depending on the extent of

a nonhomogeneous distribution of chromatin (*13*), the quantity of chromatin damaged can differ from cell to cell. Second, parts of the chromatin can be of minor or major importance for proliferation. These parts may be spatially separated in the nucleus (*14*). The possible concentration of thymine dimers in the irradiated chromatin is discussed in Section (e).

4. Due to the aperture angle of the objective used for irradiation (see Materials and Methods) the irradiated area of the nuclear envelope, and hence the energy density there, varies considerably with the position of the focus within the nucleus. The function of the nuclear envelope might, therefore, be disturbed locally to a different extent in different cells.

Since the nucleolus, which plays a special role with regard to uv-sensitivity (*4*, *15*, *16*), was excluded as a target in our present experiments, it will also be excluded from this discussion. Regarding the possible effects of uv-damage to chromatin, nuclear envelope and nucleoli, one should remember that other "nucleus-specific" bodies (e.g., interchromatin and perichromatin granules), whose functional roles are not defined (*17*), might also contribute to uv-sensitivity. Which of the points mentioned above are of real importance with regard to the heterogeneity of cell growth observed in our experiments is not clear.

(e) Since differences of irradiation energies used in our experiments were large in comparison with the relatively small differences of uv-power (Table I), the latter are omitted in the following considerations. Determination of absolute absorbed energies and energy densities is difficult in uv-microirradiation experiments. Therefore, a comparison of the absolute data given in this paper with data obtained from the literature must be made with caution. Furthermore, different ambient temperatures used during irradiation may complicate such a comparison. In spite of this, an attempt is made, assuming that the orders of magnitude may be comparable. In the paper of Smith and Dendy (*4*), an absorbed energy of $0.5 \times 10^{-4}$ ergs/$\mu m^3$ is given for the experiments carried out with an irradiation time of 0.2 sec for irradiation of the nucleoplasm. This energy was clearly sufficient to disturb cell growth. Further details concerning energy measurements are not given. Under the assumption that the absorbing volume corresponds to a cylinder 3.5 $\mu$m in diameter and with a height of 4 $\mu$m, equal to the thickness of a flattened interphase cell, an absorbed energy of approximately $2 \times 10^{-3}$ ergs per cell results. In our experiments, irradiation of the nucleoplasm clearly influenced cell proliferation when an energy of 0.01 to 0.02 FE per cell was used (Tables I and II, Figs. 2a and 3). This energy is equivalent to an absolute energy of 1 to 2 $\times 10^{-3}$ ergs per cell, assuming an absorption of 50% by the nucleoplasm. This value for the absorbed energy is in rather good agreement with the absorbed energy derived from the statement of Smith and Dendy (*4*).

Following whole-cell irradiation of various Chinese hamster cell lines at energy densities of approx $10^2$ ergs/$mm^2$, 37% (1/e) of the irradiated cells are able to form clones (*18*). This corresponds to an energy of $5 \times 10^{-2}$ ergs delivered per cell, assuming a cell area of 500 $\mu m^2$ (*19*), and 1 to $2 \times 10^{-2}$ ergs delivered per nucleus assuming a nuclear area of 100–200 $\mu m^2$. When 0.1 FE equivalent to $2 \times 10^{-2}$ ergs were delivered to the nucleoplasm by laser uv-microirradiation, we observed no proliferation (Fig. 5). This comparison tends to suggest that an energy suffici-

ent to kill practically all cells irradiated locally in the nucleus in a considerable percentage does not destroy the ability of cells to form clones when delivered to the whole nucleus, although irradiation of the whole nucleus includes the even more uv-sensitive nucleoli (*4*). To verify this suggestion, however, a direct comparison of the survival of cells of the same stock after partial and total irradiation of nuclei with uv-light of the same wavelength is required, using identical culture conditions.

A fact which deserves further investigation is the difference in the distribution of photoproducts within the nucleus: In the case of uv-microirradiation, local DNA-repair is found limited to the irradiation site [(*7*, *8*); Zorn, Cremer, and Cremer, unpublished results; see point (a)], while DNA-repair in the case of totally irradiated cells is distributed over the whole nucleus (*20*). This might influence the efficiency of DNA-repair. An energy of $2 \times 10^{-3}$ ergs (0.01 FE) corresponds to an energy density of approx $10^4$ ergs/mm$^2$ when it is focused on an area with a diameter of 0.5 $\mu$m. However, the energy density which is actually given in the focus within a living cell may differ considerably from this value: Cell structures outside the focus scatter and absorb uv-light. Furthermore, the distribution of intensity within the focus of an objective with a high aperture is highly heterogenous (*21*). But we assume that the actual mean energy density within the focus is not decreased so much that the following estimation, which suggests that there may be a strong accumulation of thymine dimers at the irradiation site, becomes invalid. Results from whole-cell irradiation of Chinese hamser cells, performed with a wavelength of 2537 Å and energy densities up to $10^4$ ergs/mm$^2$, have shown a linear increase of the dimer/thymine percentage of approx $3 \times 10^{-2}$% per 100 ergs/mm$^2$ (*22*). Assuming a similar relation in uv-microbeam experiments, a focal energy density of $10^4$ ergs/mm$^2$ would be equivalent with a dimer/thymine percentage of 3% in the focal region. However, thymine dimers should be regarded only as an example of the many possible local accumulations of photoproducts; other kinds of dimers and crosslinkings of DNA to protein (*23*) might also accumulate at the irradiation site.

(f) In uv-microbeam work with conventional uv-sources, isothermic changes are assumed to take place (*3*). The maximum focal intensities (ergs/($\mu$m$^2$sec)) used in the laser uv-microbeam experiments reported here, however, are higher by several orders of magnitude than those used in earlier work. Therefore, the question arises whether thermal effects might influence the proliferation of laser uv-microirradiated cells.

The temperature rise in the focus was estimated with the aid of a greatly simplified model (see Appendix). However, the assumed conditions were selected in such a way that the temperature rise calculated should constitute an upper limit. The conditions are as follows.

1. Because the local temperature rise increases if the focus becomes smaller, the smallest spot observed, having a diameter of 0.4 $\mu$m (*1*), is used for the calculation. The focus, therefore, is assumed to be represented by a cylinder having a diameter and a height of 0.4 $\mu$m. The whole energy incident to the cell ($E_{\text{inc}}$) is assumed to be converted into heat within this cylinder.
2. The heat conductivity of water is taken both for cell and medium.

3. Dissipation of heat occurs only perpendicular to the cylinder axis, which is coaxial with the optical axis of the uv-beam.

4. The ambient temperature at a distance of 100 $\mu$m from the focus is not perceptibly different from the temperature prior to irradiation (22°C).

At first, the stationary case was considered, resulting from a permanent uv-irradiation with the largest incident uv-power applied (0.24 ergs/sec corresponding to 1.2 FE/sec; see Table I). From these assumptions, the temperature difference between the edge of the cylinder and the temperature at a distance of 100 $\mu$m (ambient temperature) was calculated to be smaller than 0.13°C. The temperature difference between the maximum temperature obtained in the cylinder axis and the edge of the cylinder is calculated to be smaller than 0.01°C.

The temperature increases estimated for the stationary case are not exceeded in the nonstationary case (no permanent irradiation) if the heat conductivity is not decreased and if no heat-producing reaction is induced. Because there is no evidence for the induction of such reactions, a temperature rise of 0.14°C should also be an upper limit for the nonstationary case. Other models, e.g., representation of the focus by a sphere with a diameter of 0.4 $\mu$m, with heat dissipation in all directions, result in still smaller increases. Since all irradiation experiments were performed at 22°C ambient temperature, it seems very unlikely to us that such a small temperature rise should influence cell proliferation. This does not deny, however, that with the use of much higher uv-intensities [obtained, e.g., by pulsed laser sources (*3*)], thermal effects may play an important role.

## APPENDIX

### *Local Temperature Rise by Laser UV-Microirradiation*

The maximum local temperature rise is obtained in the focus of the beam. In this model, designed for the estimation of an upper limit, the focus is represented by a cylinder having a radius $R$ and a height $H$. The quantity of heat, $f$, produced in this focal cylinder per unit of volume and time, is assumed to be constant during irradiation. For $f$, an upper limit $f_0$,

$$f < f_0 := \max (E_{inc}/t_{irr})/\min V_C \qquad (1)$$

exists, max $(E_{inc}/t_{irr})$ being the largest incident uv-power (ergs/sec) applied to the cell and min $V_C$ being the smallest volume of focus used. In Table I, a largest uv-power of 1.2 FE/sec corresponding to $E_{inc} = 0.24$ ergs/sec is stated. The minimum focus diameter is given by $2R = 0.4$ $\mu$m (*1*). Inserting these figures in Eq. (1), $f_0 = 1.2 \times 10^5$ cal/(sec cm$^3$) results. The heat conductivity $k$ is assumed to be constant in cell and medium and to be equal that of water at 20°C [$k = 1.4 \times 10^{-3}$ cal/(cm sec °C)]. The temperature $T_P$ prior to irradiation is assumed to be the same in cell and medium. The dissipation of heat is assumed to occur only perpendicular to the cylinder axis, which is coaxial with the optical axis of the beam. This means that the dissipation from the top and from the bottom of the cylinder is not taken into consideration.

From the assumptions made, the differential equation for the temperature distribution (*24*) has to be solved under the following boundary conditions: $f(r, t)$

= const for $r \leq R$ and $t_0 \leq t \leq t_1$; else $f(r, t) = 0$ ($r$, distance from axis of cylinder; $t$, time). The maximum temperature rise is given for very long durations of irradiation $t_1 - t_0$. This case is characterized by a temporally constant temperature distribution. Then the following temperature distribution results:

$$T(r) = T(R) + (f/4k)(R^2 - r^2) \qquad \text{for } r \leq R, \tag{1a}$$

$$T(r) = T(R) + B \ln (R/r) \qquad \text{for } r \geq R, \tag{1b}$$

where $T(R)$ is the temperature at the cylinder surface. The numerical value for $B$ is calculated from common equilibrium considerations to be smaller than $B_0 = 1.6 \times 10^{-2}$ °C, even if one assumes that the largest incident uv-power applied to the cell is absorbed totally within the focus cylinder. It seems reasonable to assume that under the actual conditions used, for $r = 100$ μm the temperature ($T_{100}$) does not change significantly compared to the temperature prior to irradiation ($T_P$):

Inserting $B_0$ into Eq. (1b), $T(R) - T_P \approx T(R) - T_{100} < 1.3 \times 10^{-1}$ °C is calculated. The temperature distribution inside the cylinder is given by Eq. (1a). As Eq. (1a) shows, the maximum temperature is obtained for $r = 0$. With $f_0 = 1.2 \times 10^5$ cal/(sec cm³), $T(r = 0) - T(R) < 1 \times 10^{-2}$ °C follows. Hence, the maximum temperature rise which could occur under the assumptions made is $T(r = 0) - T_{100} < 0.14$°C.

The temperature rises actually induced are probably still lower. If one assumes that only a small part ($\lesssim 10\%$) of the total absorbed energy is absorbed in the focal region itself, a maximum temperature rise of the order of magnitude $10^{-2}$ °C follows from the equations above. A still smaller value results if instead of a cylindrical focus a model is considered in which the focus is represented by a sphere of 0.4 μm diameter and heat is dissipated in all directions. In this case, a maximum temperature rise of the order of magnitude $10^{-3}$ °C results.

## ACKNOWLEDGMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB 46). The cell line used was obtained from Dr. D. Wild. For helpful and stimulating discussions, we are greatly indebted to Professors W. Krone, Ch. Streffer, and U. Wolf. We are particularly grateful to Mr. J. Zimmer for skillful technical assistance.

RECEIVED: August 1, 1975; REVISED: October 15, 1975

## REFERENCES

1. C. CREMER, C. ZORN, and T. CREMER, An ultraviolet laser microbeam for 257 nm. *Microsc. Acta* **75**, 331–337 (1974).
2. G. MORENO, M. LUTZ, and M. BESSIS, Partial cell irradiation by ultraviolet and visible light: Conventional and laser sources. *Int. Rev. Exp. Pathol.* **7**, 99–137 (1969).
3. G. MORENO, C. SALET, and M. BESSIS, Microirradiation de noyaux cellulaires par rayonnement laser ultraviolet. *C. R. Acad. Sci. Paris, Ser. D* **269**, 781–782 (1969).
4. C. L. SMITH and P. P. DENDY, Disturbances in the development of small colonies following the irradiation of different parts of single cells in tissue culture with a heterochromatic microbeam of ultraviolet light. *Cell Tissue Kinet.* **1**, 225–237 (1968).
5. C. ZORN, C. CREMER, and T. CREMER, A continuous-wave ultraviolet laser microbeam and its use for studies in somatic cell genetics. In *Proceedings of the Technical programme.*

*Electro-Optics International '74 Conference*, Brighton, England, March 19–21, 1974 (M. S. Kiver, Ed.), pp. 203–210. Kiver Communications, Surrey, 1974.

6. E. H. Y. Chu, P. Brimer, K. B. Jacobson, and E. V. Merriam, Mammalian cell genetics. I. Selection and characterization of mutations auxotrophic for 1-glutamine or resistant to 8-azaguanine in Chinese hamster cells in vitro. *Genetics* **62**, 359–377 (1969).
7. G. Moreno, Effects of ultraviolet micro-irradiation on different parts of the cell. II. Cytological observations and unscheduled DNA synthesis after partial nuclear irradiation. *Exp. Cell. Res.* **65**, 129–139 (1971).
8. G. Moreno and Ch. Salet, Unscheduled DNA synthesis after ultraviolet micro-irradiation of the cell nucleus. *Radiat. Res.* **58**, 52–59 (1974).
9. J. M. R. Hatfield, L. Schulze, and D. Ernst, Measurement of the ultraviolet absorption in specific parts of both living and fixed mammalian cells, using a specially designed microspectrophotometer. *Exp. Cell Res.* **59**, 484–486 (1970).
10. M. M. Elkind and G. F. Whitmore, *The Radiobiology of Cultured Mammalian Cells*. Gordon and Breach, New York, 1967.
11. W. K. Sinclair and R. A. Morton, X-ray and ultraviolet sensitivity of synchronized Chinese hamster cells at various stages of the cell cycle. *Biophys. J.* **5**, 1–25 (1965).
12. H. Kato, Differential chromosomal sensitivities of aneusomic cell clones to uv light. *Exp. Cell Res.* **83**, 55–62 (1974).
13. K. Brasch and G. Setterfield, Structural organization of chromosomes in interphase nuclei. *Exp. Cell Res.* **83**, 175–185 (1974).
14. R. B. Painter, B. R. Young, and H. J. Burki, Non-repairable strand breaks induced by $^{125}$J incorporated into mammalian DNA. *Proc. Natl. Acad. Sci. USA* **71**, 4836–4838 (1974).
15. I. Deak, E. Sidebottom, and H. Harris, Further experiments on the role of the nucleolus in the expression of structural genes. *J. Cell Sci.* **11**, 379–391 (1972).
16. J. M. R. Hatfield, P. P. Dendy, F. Meyskens, and A. B. Rickinson, Importance of the nucleolus in the initiation of DNA synthesis in mammalian cells. *Exp. Cell Res.* **78**, 214–224 (1973).
17. H. Busch (Ed.), *The Cell Nucleus*, Vol. I. Academic Press, New York/London, 1974.
18. R. B. Painter, The action of ultraviolet light on mammalian cells. *Photophysiology* **5**, 169–189 (1970).
19. E. H. Y. Chu, Effects of ultraviolet radiation on mammalian cells. I. Induction of chromosome aberrations. *Mutat. Res.* **2**, 75–94 (1965).
20. For example, R. B. Painter and J. E. Cleaver, Repair replication, unscheduled DNA synthesis, and the repair of mammalian DNA. *Radiat. Res.* **37**, 451–466 (1969).
21. R. Barakat, Vector diffraction theories. In *Progress in Optics* (E. Wolf, Ed.), Vol. I, pp. 99–104. North-Holland, Amsterdam, 1965.
22. D. L. Steward and R. M. Humphrey, Induction of thymine dimers in synchronized populations of Chinese hamster cells. *Nature (London)* **212**, 298–300 (1966).
23. P. Alexander and H. Moroson, Cross-linking of deoxyribonucleic acid to protein following ultraviolet irradiation of different cells. *Nature (London)* **194**, 882–883 (1962).
24. W. Weizel, *Lehrbuch der Theoretischen Physik*, Vol. I, pp. 795 ff. Springer–Verlag, Berlin, 1963.