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Genetic Microsurgery by Laser: Establishment of a Clonal Population of Rat Kangaroo Cells (PTK₂) with a Directed Deficiency in a Chromosomal Nucleolar Organizer

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Abstract. An ultraviolet laser beam was focused to a submicron spot on one of the nucleolar organizer regions of mitotic chromosomes of rat kangaroo cells in tissue culture. The daughter cells were isolated and cloned into a viable population that maintained the directed nucleolar deficiency. It is concluded that the laser can be used to delete preselected genetic regions and the genetic deletion is maintained as a heritable deficiency in subsequent daughter cells.

Introduction

In 1969, it was reported that the green beam of an argon laser could be focused to a spot of less than 1 μm on preselected arms of mitotic chromosomes in tissue culture cells (Berns et al., 1969). The cells were from primary tissue cultures of salamander lung, and the chromosomes had been pretreated with acridine orange to facilitate absorption of the laser light. These early experiments demonstrated that it was possible to selectively delete chromosome segments as small as 0.5 μm in living cells while maintaining post-irradiation cell survival for several days. Subsequent studies (Berns et al., 1970; Berns and Cheng, 1971) demonstrated that it was possible to selectively irradiate the nucleolar organizer region of the chromosome and thereby produce post-mitotic cells that were deficient in various numbers of nucleoli (depending upon how many nucleolar organizer regions were irradiated). However, in none of our early studies was it possible to establish viable cell clones maintaining the induced genetic deficiency. The reason for this was twofold: (1) the acridine orange plus laser treatment resulted in DNA and protein damage in the chromosomes far too severe for the maintenance of long term cell viability, (2) single cell clones could not easily be produced from salamander lung cultures because they were primary cultures and therefore had a very low cloning efficiency.

To minimize the damage severity, a tunable wavelength laser microbeam system was developed (Berns, 1972) which provided wavelengths at a wide

variety of energy densities from the ultraviolet (240 nm) through the entire visible portion of the spectrum. With this laser system, it was possible to produce selective chromosome lesions without the use of any vital stain (Berns, 1974a) when either ultraviolet *or* visible wavelengths were used.

The cell cloning problem was approached by replacing the primary cultures with a suitable established cell line. The cell line PTK₂ (from the male rat kangaroo, *Potorous tridactylis*) was selected because it possesses several features which facilitate laser irradiation and subsequent analytic techniques, such as electron microscopy and karyotyping (Berns, 1974b). The cells are near diploid, they have a low chromosome number, and they remain relatively flat during mitosis, thus making the cells' chromosomes readily visible. The cells have a single nucleolus organizer region, located on the X chromosome, which can be identified and microirradiated. The resulting post-mitotic cells from such an irradiation do not produce any normal nucleoli nor are the cells capable of subsequent mitosis (Berns et al., 1972). A tetraploid subline has been established which has two X chromosomes with visible nucleolar organizer genetic regions and, therefore, two nucleoli per cell (Branch and Berns, 1976).

Using this cell line, a series of investigations was undertaken in which the laser was focused onto one of the nucleolar organizer regions in cells of the tetraploid line and the subsequent daughter cells isolated and cloned. The clonal cells have been shown to be deficient in one nucleolus; they have maintained the tetraploid condition; and they have both X chromosomes. When Giemsa banding techniques are applied to the chromosomes, it is clear that the irradiated chromosome is deficient in a small region corresponding to the site of the nucleolar genes. The results of these studies are presented, and the general applicability of selective genetic deletion by laser microsurgery is discussed.

Materials and Methods

The laser microbeam system consisted of a Chromatix #1000 neodymium-YAG laser with an output energy of 5 KW at the second harmonic wavelength of 532 nm. The green beam (532 nm) was frequency shifted to 265 nm by passage through a fixed frequency ADP second harmonic generator, Chromatix model #1050. The final laser output at 265 nm was 500–1,000 W with a pulse duration of 80 nanoseconds. Alternatively, the 532 nm laser beam was used to stimulate the dye rhodamine 6G in a tunable dye laser. The visible output of the dye laser at 560 nm was subsequently frequency doubled with an internally mounted KDP crystal, thus yielding ultraviolet laser light of 280 nm. Experiments were conducted using either 265 nm or 280 nm laser light.

The ultraviolet beam of laser light was next deflected into a Zeiss photomicroscope by an especially designed, highly UV-reflective dichroic filter. The photomicroscope was modified by replacement of the normal visible light transmitting tube head with an ultraviolet transmitting head. The laser beam was transmitted through the microscope and focused by either a 32× Zeiss Ultrafluar objective or a 100× Zeiss Ultrafluar objective to spot diameters of between 0.2 to 1.0 μm. Ultraviolet neutral density filters were used to attenuate the laser beam in order to achieve desired energy densities. Laser energy was monitored with a calibrated Eppley thermopile #14011 attached to a Hewlett Packard #419 voltmeter. Energy densities in the focused spot varied between 0.001 μJ/μm² and 0.1 μJ/μm² depending upon the experimental requirements.

Observation of the target cells was accomplished by projecting the cell image up the microscope optical path (coincident with the laser beam) and through the dichroic filter to a high light sensitivity television camera (RCA #TC100). The cell image was then projected onto a television monitor

with a crosshair depicting the point of laser focus. The target area of the cell was next moved under the crosshair on the monitor screen, and the laser was triggered by remote control. All television images were recorded on videotape, thus providing a before, during, and after record of each microirradiation experiment. The videotape system was a GYYR model DAS300 time lapse video recorder. It was therefore possible to follow the cells by time lapse videotape for several days following irradiation. Since it was critically important to determine that subsequent cells were true division products (clonal descendants) from a single irradiated cell, the immediate playback time lapse feature of the videotape system was indispensable.

The cells used in this study are from a tetraploid clonal subline that was derived from near diploid male rat kangaroo PTK₂ (modal chromosome number 12–13), *Potorous tridactylis*, cells (Branch and Berns, 1976). The tetraploid line is designated PTK₂WA and its modal chromosomal number is 24–25. This line has maintained the tetraploid condition for over three years. The nucleolar organizers are on the X chromosomes and are recognizable as secondary constrictions just below the centromeric constrictions. In both the diploid and tetraploid lines, there is a close correlation between the number of nucleoli and the number of X chromosomes: diploid PTK₂ cells have one nucleolus and tetraploid PTK₂WA cells have two nucleoli (Branch and Berns, 1976).

The cells were grown as monolayers in Rose tissue culture chambers in a modified Eagle's medium (Berns, 1978). Instead of using two #1 thickness glass coverslips for the chamber windows, one coverslip was glass and the other was quartz (thickness of 0.32 mm) manufactured by Esco Company (Oakridge, New Jersey). Cells growing on the quartz coverglass were irradiated with the UV laser beam.

Results and Discussion

Irradiation of the tetraploid PTK₂WA cells took place 2–3 days following injection of the cells into the Rose chamber. Cells were selected for irradiation when they were in early prophase. At this time, the nuclear membrane was in the process of breaking down, the chromatin was condensing, and the nucleoli were starting to disappear. The laser was focused onto one of the two nucleoli, and the cell was observed continuously with the time lapse video system. Since irradiation of the nucleolus resulted in damage to the nucleolar DNA (rDNA), it was possible to assay the result by following the cell through mitosis and observing the re-synthesis of nucleoli in the two post-mitotic daughter cells (Figs. 1–3). The experiment was repeated 36 times with 280 nm laser light and 59 times with 265 nm laser light. In the case of 280 nm, two daughter cells, each deficient in one nucleolus, were produced 4 times, and in the case of 265 nm, daughter cells with a nucleolar deficiency were produced 14 times. Subsequent isolation and cloning demonstrated that only those cells treated with the 265 nm light were able to continue through additional mitosis. A clone of cells maintaining the nucleolar deficiency was derived from a single cell in which one nucleolar genetic region was irradiated (Fig. 1).

The isolation and cloning procedure was similar to that devised for cells that had entire chromosomes removed by irradiation of centromere regions (Berns, 1974c). When the clonal descendants of the original cell had reached confluency, the culture was subcultured until enough cells were available for freezing in liquid nitrogen. Following establishment of a frozen stock, the clonal cells still growing in the T₃₀ flasks were analyzed for DNA content and chromosome number.

Since there was a remote possibility that the single nucleolar cells were

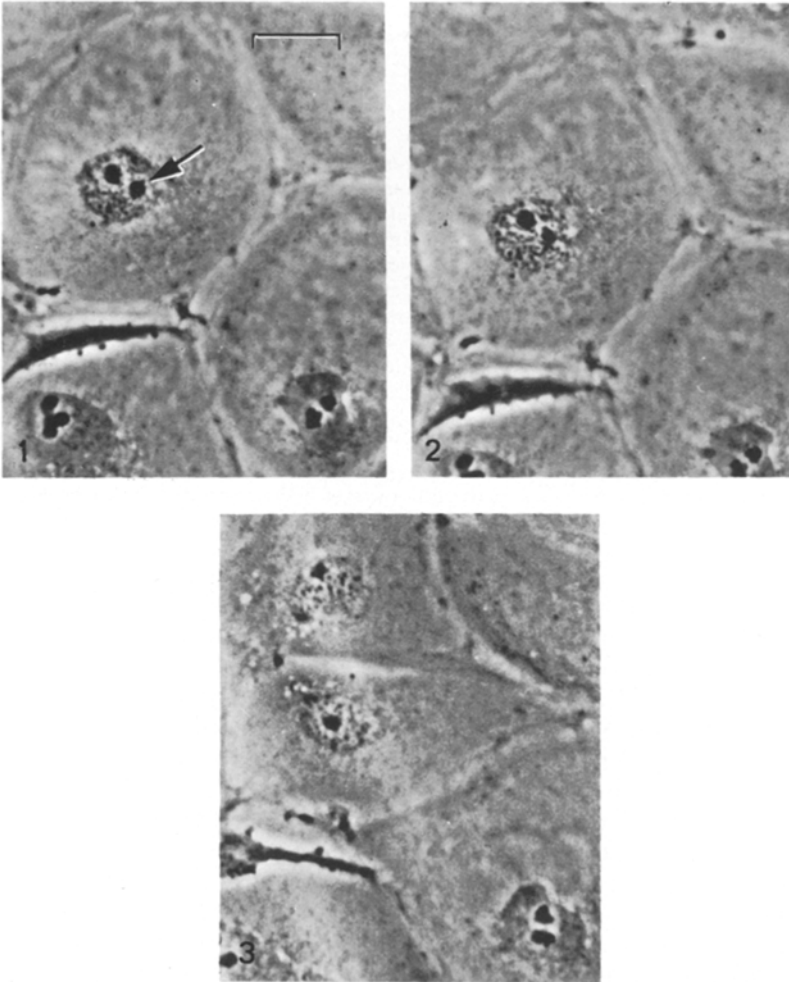


Fig. 1. Tetraploid PTK₂ cell just prior to irradiation of one nucleolus in early prophase. Note the comparison between the condensing chromatin of the cell in this photo and Fig. 2. The arrow indicates the nucleolus that will be irradiated. Bar=10 μm

Fig. 2. Immediately post-irradiation; note the slight change in morphology of the irradiated nucleolus. The entire nucleolus was irradiated

Fig. 3. The two daughter cells approximately 2 h after irradiation. Note that both cells clearly have only one nucleolus. These two cells subsequently divided giving rise to an entire population of cells with one nucleolus

really derived from contaminating, diploid PTK₂ cells, studies to define the ploidy level of the clonal population were undertaken. This type of analysis was further necessitated because in earlier studies (Brenner et al., 1977), we had some evidence that PTK₂ cells of higher ploidy (tetraploid, hexaploid, octaploid, etc.) could undergo an unusual two step, meiotic-like reduction divi-

sion *in vitro*. It, therefore, seemed possible that the single nucleolar clone was diploid rather than tetraploid. Quantitative determination of DNA content by fluorescence analysis was undertaken to answer the question of ploidy level.

Quantitative DNA measurements were made on individual cell nuclei of three cell populations: (1) the nucleolar deficient clone (PTK₂ clone), (2) the parental tetraploid population (PTK₂WA), and (3) the original diploid population from which the tetraploid population was derived (PTK₂).

Cells were stained by the standard Feulgen-Schiff reaction (Leuchtenberger, 1958). When observed with regular bright field light microscope optics, the nuclei and chromosomes appear red. In the past, quantitative DNA determinations generally have involved making absorption measurements on cells stained in this manner because the Feulgen-Schiff stain does not provide high levels of fluorescence when illuminated with the standard mercury lamps. However, in the studies reported here, we employed the 440 nm wavelength of a helium-cadmium laser to stimulate fluorescence in the Feulgen stained cells. By combining the laser with a Nanometrics Nanospec 10 microspectrofluorometer, it was possible to generate very strong fluorescent signals from the Feulgen stained cells (Berns, *in press*). Over 100 cell nuclei from each of the three cell populations were measured by the above method. From the data presented in Figure 4, it is clear that the nucleolar deficient clone (PTK₂ clone) is tetraploid rather than diploid.

Even though the preceding data clearly show that the clonal population is tetraploid, it does not provide specific information about the chromosome complement.

The level of sensitivity of the fluorescence system would not be able to discriminate between a tetraploid cell with two X chromosomes and a near tetraploid cell with only one X chromosome. It is important to make this distinction because selective irradiation of one X chromosome could have resulted in that entire chromosome not being replicated at the subsequent S phase of the cell cycle. A clone derived from such a cell would still be tetraploid with respect to all its chromosomes except the X. Such a cell would have only one nucleolus. The other alternative would be a situation where irradiation of the X chromosome resulted in damage only to the secondary constriction region of the chromosome. Clonal cells would be expected to have two X chromosomes but only one of the Xs would have a functional nucleolar organizer, and therefore, the cells would have only one nucleolus. Karyotype analysis was undertaken to resolve this question.

Initial karyotype analysis employing non-banded, Feulgen stained cells revealed that the clonal population was tetraploid. However, the cells had only one clearly identifiable X chromosome. Another X-like chromosome was present, but it did not have a secondary constriction.

A modified Giemsa banding technique (Seabright, 1972) was used in order to more precisely identify the X-like chromosome. Cells were blocked for 6 h to overnight in 0.06 µg/ml colcemid, swollen with 0.075 M KCl, fixed with acetic acid:methanol (3:1), and dropped onto slides. After air drying for several days, the preparations were treated with 0.05% trypsin (Difco 1:250) in sodium citrate buffer for 20–60 sec, rinsed with 0.09% saline (2X), stained with 3% Giemsa (Harleco Azure A) in dilute McIlvaine's buffer, rinsed in dilute buffer,

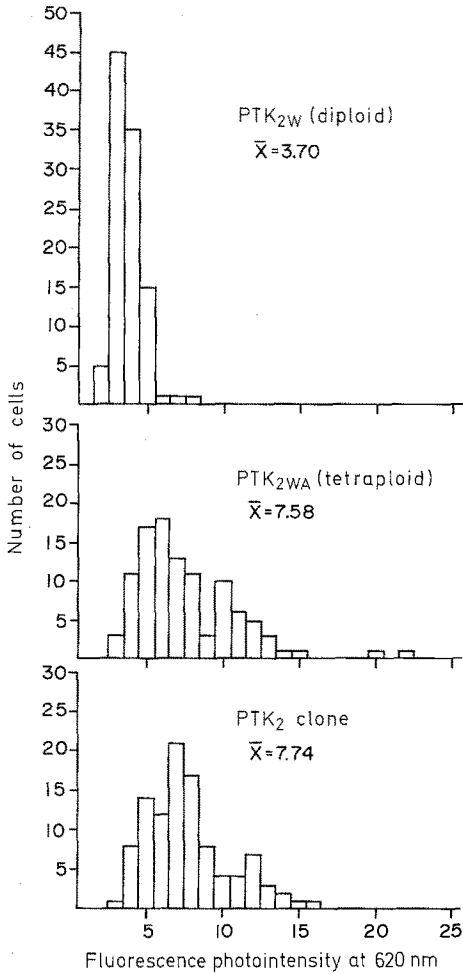


Fig. 4. Quantitative fluorescent Feulgen measurements of the nuclear DNA content of the diploid (PTK₂W), tetraploid (PTK₂WA) and the clonal populations. Both the distribution and mean values of the clone and tetraploid cells are very similar. It is concluded that the single nucleolar clone is tetraploid rather than diploid

air dried and coverslipped with Permount. Chromosome spreads were located and photographed on a Zeiss photomicroscope III with a $63\times$ oil objective using Kodak high contrast copy film. Ten clearly banded, well spread karyotypes were carefully examined. (Three are presented in Fig. 5). Nine of the ten exhibited two X chromosomes and clearly demonstrated that the unidentified chromosome was really the second X. The banding patterns of the large chromosome groups (Nos. 1, 2 and 3) were very distinctive amongst themselves as well as entirely different from the banding pattern of the X chromosome. Furthermore, upon careful examination of the two X chromosomes, it appeared that (Fig. 5) there was a difference between the X chromosomes in the region of the secondary constriction. One of each X was missing a light band in the region of the nucleolar organizer (Arrows, Fig. 5). This region corresponded closely to the region deleted with the laser.

The studies described in this paper indicate that the technology now exists to

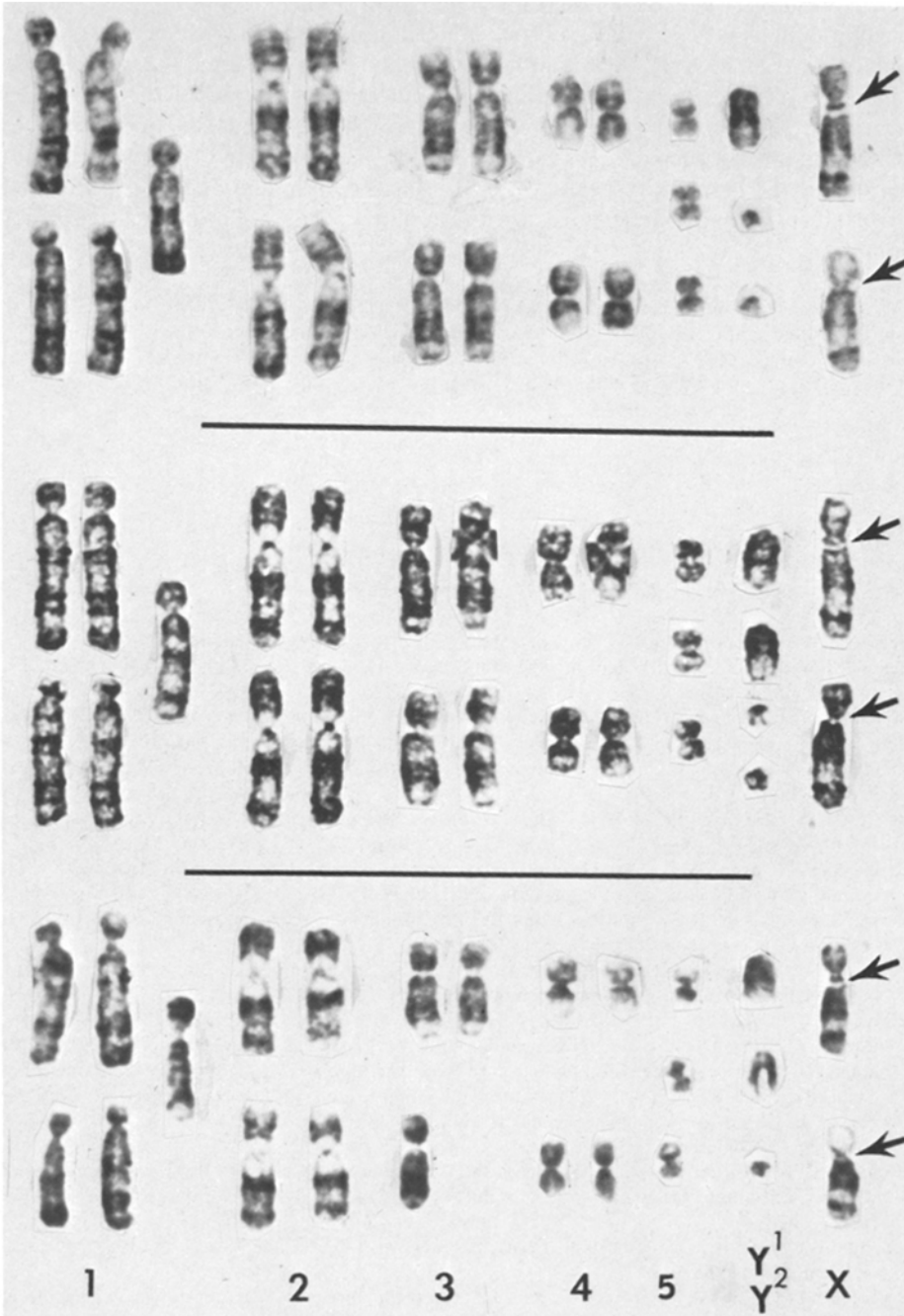


Fig. 5. Three Giemsa banded karyotypes of the clone. The banding patterns are distinct for each of the chromosome pairs. Of particular importance is the determination that there are two X chromosomes. Furthermore, one of the two X chromosomes in each karyotype is missing a band in the secondary constriction region (*arrows*)

delete preselected genetic regions of individual chromosomes and that the deletion is maintained as such over subsequent cell generations. With respect to the nucleolar organizer genes, it is now possible to selectively alter this gene group in tissue culture cells. This capability should permit studies on the ribosomal genes that in the past have been confined to the classical genetic systems in whole organisms, such as *Xenopus* and *Drosophila*. However, the broader implications of the results presented here are that the laser microbeam can be used to produce heritable deficiencies on preselected regions of individual chromosomes.

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References

- Berns, M.W.: Partial cell irradiation with a tunable organic dye laser. *Nature (Lond.)* **240**, 483-485 (1972)
- Berns, M.W.: Recent progress in laser microbeams. *Int. Rev. Cytol.* **39**, 383-411 (1974a)
- Berns, M.W.: Laser microirradiation of chromosomes. *Cold Spr. Harb. Symp. quant. Biol.* **38**, 165-174 (1974b)
- Berns, M.W.: Directed chromosome loss by laser microirradiation. *Science* **186**, 700-705 (1974c)
- Berns, M.W.: The laser microbeam as a probe for chromatin structure and function. In: *Methods in cell biology*, vol. 18 (G. Stein, J. Stein and L. Kleinsmith eds.), p. 277. New York: Academic Press Inc. 1978
- Berns, M.W.: Fluorescent analysis of cells using a laser light source. *Cell Biophysics* (in press 1979)
- Berns, M.W., Cheng, W.K.: Are chromosome secondary constrictions nucleolar organizers: a re-evaluation using a laser microbeam. *Exp. Cell Res.* **69**, 185-192 (1971)
- Berns, M.W., Olson, R.S., Rounds, D.E.: In vitro production of chromosomal lesions using an argon laser microbeam. *Nature (Lond.)* **221**, 74-75 (1969)
- Berns, M.W., Ohnuki, Y., Rounds, D.E., Olson, R.S.: Modification of nucleolar expression following laser micro-irradiation of chromosomes. *Exp. Cell Res.* **60**, 133-138 (1970)
- Berns, M.W., Floyd, A.D., Adkisson, K., Cheng, W.K., Moore, L., Hoover, G., Ustick, K., Burgott, S., Osial, T.: Laser microirradiation of the nucleolar organizer in cells of the rat kangaroo (*Potorous tridactylis*): reduction of nucleolar number and the production of micronucleoli. *Exp. Cell Res.* **75**, 424-432 (1972)
- Branch, A.B., Berns, M.W.: Nucleoli and ploidy in *Potorous* cells (PTK₂) in vitro. *Chromosoma (Berl.)* **56**, 33-40 (1976)
- Brenner, S., Branch, A., Meredith, S., Berns, M.W.: The absence of centrioles from spindle poles of PTK₂ cells undergoing meiotic-like reduction division in vitro. *J. Cell Biol.* **72**, 368-379 (1977)
- Leuchtenberger, C. In: *General cytochemical methods*, vol. 1 (J.F. Danielli, ed.), p. 219. New York: Academic Press Inc. 1958
- Seabright, M.: The use of proteolytic enzymes for the mapping of structural rearrangements in the chromosomes of man. *Chromosoma (Berl.)* **36**, 204-210 (1972)