Directed Chromosome Loss by Laser Microirradiation

By means of a laser microbeam an entire chromosome or a small preselected area of a chromosome can be deleted.

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Recent progress in somatic cell genetics has demonstrated the importance of generating and characterizing new mutant cell lines. One of the ultimate goals in this field is the assignment of specific genes to chromosomes and eventually to particular loci. Various new approaches have been developed with these objectives in mind. One of the most useful has been the fusion of cells from different organisms, such as mouse cells and human cells. As a result of the fusion, the chromosomes of both cells are contained within one cytoplasm. This process is followed by the random loss of the human chromosomes from the mouse cell. When this procedure is combined with classical genetic approaches, such as screening for nutritional growth requirements, temperature sensitivity, or protein differences, it is possible to construct linkage maps and to assign specific genes to various chromosomes. However, the generation and detection of new mutants is difficult because of the abnormal ploidy of most cell lines. The detection of large numbers of mutations is extremely difficult, if not impossible, with these techniques.

Investigators have used a variety of approaches in attempts to alleviate this situation, including: (i) the development of haploid cell lines (*1*), and (ii) the selective disruption of the mitotic apparatus by micromanipulation (*2*). Both of these approaches have proved difficult. In addition, the classical method of inducing genetic variation by way of mutation involves the application of the mutagenic agent (a chemical, or radiation) to a whole population of cells. Such treatment produces an undefined class of mutations in interphase cells. Subsequent isolation of the mutants by colonial growth can, at best, only uncover a small percentage of the mutations. Because of the randomness of this approach and, of course, the lethality of many mutations, it may never result in the isolation of mutants for specific chromosomes or chromosome regions.

The most direct approach would be to preselect a chromosome and either delete it entirely or cause a small region of it to mutate. Then one could determine whether or not the cell, in the absence of all or part of that chromosome, could be cloned, and if so, one could perform subsequent growth, biochemical, and karyological analyses on the descendant population. This approach would (i) facilitate the assignment of genes to chromosomes; (ii) make it possible to determine which chromosomes and chromosome regions are essential for immediate cell survival or survival through several cell cycles under maximal growth conditions; (iii) generate new classes of mutants; (iv) permit direct analysis of chromosome stability and DNA constancy in variant cell lines; and (v) facilitate investigation of the repair of chromosomal damage at varying times after the mutational event (after one cell cycle, two cell cycles, and so on). In this laboratory we are attempting to accomplish these objectives using laser microbeam irradiation of preselected chromosomes.

The Laser Microbeam Method

Microbeam irradiation has been used extensively since 1912 in studies on cell function (*3*). The essential feature of this technique is that the beam of damaging radiation (electromagnetic or particulate) is focused onto a specific cell organelle or cell region. In the optimal situation, the damage produced by the radiation is limited to the target. Numerous studies on both nuclear and cytoplasmic structures have been conducted with microbeams of ultraviolet radiation, x-rays, electron beams, and proton beams, for example (*4, 5*).

Until recently, the technique of microirradiating chromosomes had been used only in the early studies of Zirkle (*4*), who described the phenomenon of chromosome "paling" following ultraviolet microirradiation, and in studies of several investigators (*6*) who used ultraviolet microbeams to irradiate various parts of the mitotic apparatus. Though many of these investigators described abnormal chromosome movements and perturbations in the mitotic process, none of them attempted to isolate and follow the cells for a prolonged period of time. To do so would have been difficult because of the secondary effects of exposure to ultraviolet radiation; the duration of such exposure was often measured in minutes. In addition, the mechanics of the ultraviolet microbeam required the use of quartz or reflecting optics and special culture chambers fitted with a quartz window.

When the argon laser beam became available in 1968, a microbeam system was constructed for use in chromosome studies. Chromosome paling was subsequently described in tissue culture cells of the salamander *Taricha* that were exposed to this laser beam (*7*). The beam was of low power (1.5 watts), of short duration (50 microseconds), and contained two primary visible wavelengths (514 and 488 nanometers). To produce the chromosome paling, the cells had to be treated with a solution of acridine orange (1 to 0.01 microgram per milliliter) for 5 minutes prior to the laser irradiation. More powerful microbeams were then constructed, one with a 35-watt pulsed argon laser (*8*) and another with a 1-megawatt organic dye laser (*9*). By using the blue and green wavelengths from either of these devices, it was possible to produce the chromosome paling without any acridine orange treatment (*10*). The duration of radiation exposure in all cases varied from 10^{-7} to 10^{-5} second. The paling spot

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on the irradiated chromosome varied from 0.5 to 2 micrometers in diameter, depending upon the laser energy and the optics employed.

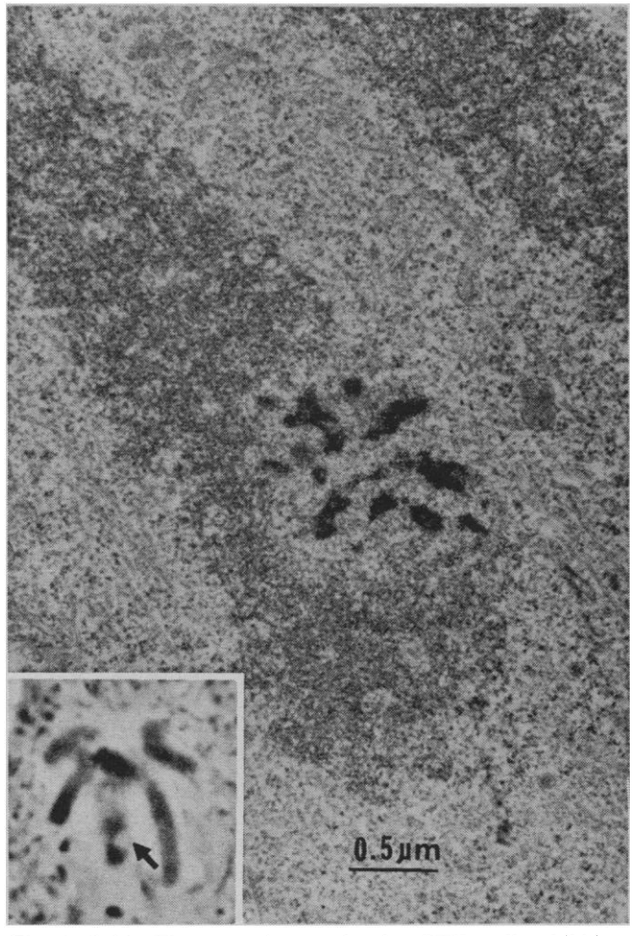
Both the argon and the dye lasers are now used in chromosome studies. The two systems are combined with closed circuit televisions equipped with time-lapse videotape (*11*) and they are mounted above Zeiss photomicroscopes.

The cells are grown in standard Rose culture chambers and are viewed through the microscope by the phase-contrast technique. The image of the target mitotic cell is projected on the TV monitor screen. By carefully moving the mechanical stage of the microscope, a desired region of a target chromosome is moved under a cross hair on the monitor screen. When the laser is fired, the beam is focused by a $\times 100$ phase Neofluar objective to a small spot within the cell (0.25 to 1 μm in diameter). During the entire procedure, the cell is videotaped and photographed.

Chromosome Lesions

The cells used in the studies described in this article were rat kangaroo kidney cells of the PTK_2 (*Potorous tridactylis*; $2n = 13$) cell line (American Type Tissue Culture Collection CCL 56). This is an XYY cell line that is trisomic for the large, acrocentric chromosome No. 1. All of the studies described herein involved irradiation of this chromosome. The natural flattening of PTK_2 cells during mitosis is ideal for recognition of chromosomes and aiming the microbeam. The same flattening effect can be attained in the more rounded cells of mammals by gently stretching a strip of sterile dialysis membrane across the bottom cover slip of the culture chamber.

By using a laser microbeam it is possible to damage a small preselected area of a chromosome. Irradiation of the long arm of chromosome No. 1 with at least 1000 microjoules per square micrometer for 10^{-6} second



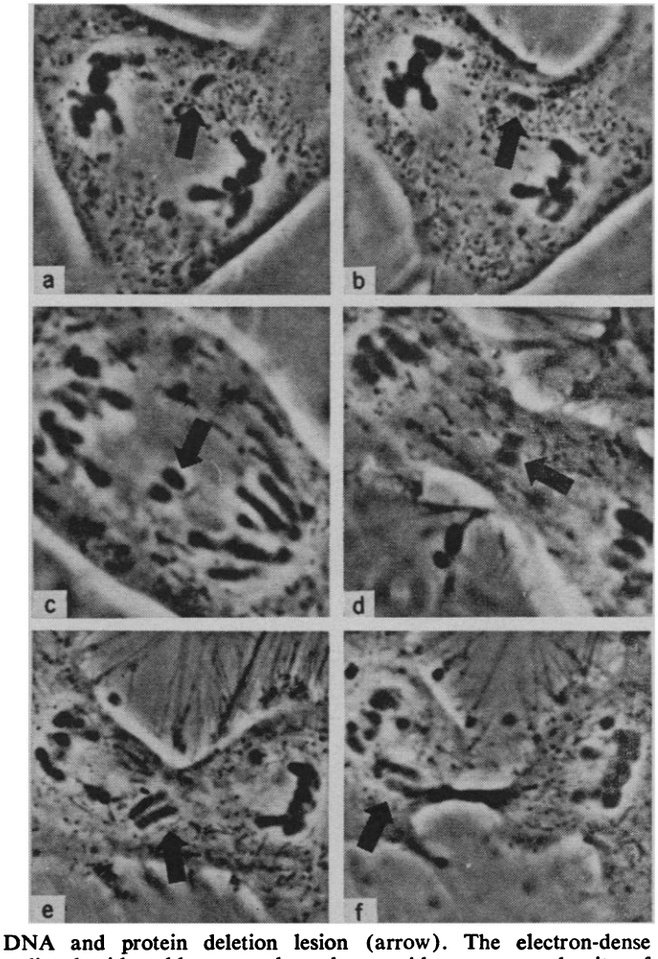


Fig. 1 (left). Electron micrograph of a PTK_2 cell exhibiting a DNA and protein deletion lesion (arrow). The electron-dense area represents the damaged material. This chromosome was irradiated with a blue-green laser beam with an energy density of 1000 microjoules per square micrometer. By electron microscopy the lesion area is shown to be confined precisely to the region of the chromosome that was irradiated. This correlates with the photomicrograph (see inset) of the living cell examined by phase-contrast microscopy. Fig. 2 (right). (a and b) Anaphase cell demonstrating the loss of an irradiated chromosome from the nucleus. The irradiated chromosome can be seen in the cytoplasm; the two light micrographs show slightly different focal planes in the cell; the two chromatids are indicated by the arrows. (c and d) Irradiated chromosomes lost during mitosis as a result of being caught in the stem body; (c) anaphase cell showing the two chromosomes remaining behind at the metaphase plate; (d) the same cell in telophase showing the two chromosomes being caught in the stem body; neither daughter cell received one of these chromosomes. (e and f) Both microirradiated chromosomes being incorporated in the nucleus of one daughter cell; (e) early telophase showing both chromosomes being incorporated in one daughter cell; (f) late telophase showing both chromosomes being incorporated into one nucleus.

Table 1. Summary of cloning results with PTK_2 cells subjected to microirradiation with a laser beam. The percentage of cells cloning is determined by dividing the number of successful clones by the total numbers of cells that we attempted to clone.

Treatment of cell	Total attempts	At least one additional mitosis	More than one additional mitosis	Successful clones	Cloning (%)
Irradiation of a chromosome*	102	37	21	9	9
Irradiation of nonchromosome region (control)	9	9	9	6	66
No irradiation (control)	26	25	24	21	80

* This group includes cells in which chromosome No. 1 was either partially deleted or was removed entirely by kinetochore irradiation.

always results in the appearance of a small "paled" spot 0.25 to 1 μm in diameter (see inset, Fig. 1). When the chromosomes are cytochemically stained by the Feulgen procedure, negative DNA staining occurs in the lesion area (*12*). Earlier studies demonstrated that the negative Feulgen staining was correlated with loss of genetic function: when the ribosomal DNA region (the secondary constriction) was irradiated, the cell lost its ability to synthesize a postmitotic nucleolus at that site (*12*). The confinement of the lesion to the paled spot was demonstrated by the fact that irradiation of the chromosome as close as 0.25 μm to a known functional site did not affect the functionality of that region. In addition, recent electron microscopic studies of the microirradiated chromosome demonstrate that the paling spot correlates precisely with ultrastructural damage (Fig. 1). Studies with the electron microscope demonstrate also that adjacent structures, such as mitochondria, microtubules, and membranes, are not affected by the laser microirradiation (Fig. 1). These observations help explain the survival of these cells and their ability to form clones.

In addition to being able to damage a preselected area of a chromosome, the laser microbeam can also be used to remove whole chromosomes from cells. If the kinetochore region of chromosome No. 1 is irradiated at metaphase, with the same laser parameters as in the preceding experiments, the irradiated chromosome is excluded from the mitotic spindle. Chromosomes have been observed (i) "falling off" the metaphase plate and drifting out into the cytoplasm (Fig. 2, a and b); (ii) remaining at the metaphase plate and subsequently remaining in the center of the cell while the other chromosomes undergo anaphase movements toward the poles—the irradiated chromosome is caught in the stem body during cytokinesis and lost from the cell (Fig. 2, c and d); (iii) moving randomly toward one pole or another—the irradiated chromosomes end up in a nucleus with the unirradiated chromosomes (Fig. 2, e and f), or they may stay in the cytoplasm and be incorporated into a micronucleus. With the above possibilities, the resulting daughter cells may have chromosome complements of one less or one more than the normal number for the cell line.

Examination of kinetochore irradiated chromosomes by electron microscopy demonstrated that the microbeam irradiation was not always successful in hitting the kinetochore. The kinetochore is not visible with the light microscope. Consequently, when one is aiming the microbeam, the cross hair is positioned over that part of the chromosome that appears light under the phase-contrast microscope and represents the primary constriction (the centromere). It is possible that the kinetochore is not irradiated if (i) the centromeric region is considerably larger than the kinetochore, (ii) the kinetochore is not located right in the center of the centromere, or (iii) the laser is slightly off the target cross hair. In all of these cases, the chromatin near the kinetochore might be irradiated instead. Studies of serial thin sections of chromosomes suspected of having been irradiated in the area of the kinetochore support this theory. In some experiments, the kinetochore appears to have been destroyed, and no kinetochore microtubules are detected emanating from the chromosome. In other cases, the laser damage can be seen near the kinetochore. The kinetochore appears to be intact with numerous kinetochore microtubules attached (Fig. 3). Both types of kinetochore irradiations result in chromosome loss. When the kinetochore is destroyed, there are no microtubules to attach it to the spindle, and the chromosome simply falls off. When the laser beam misses the kinetochore, apparently the chromatin around the kinetochore is so weakened that the microtubules pull the kinetochore right out of the chromosome (see Fig. 3). Viewed with the light microscope, the remaining chromosome appears to be detached from the spindle as in the previous case.

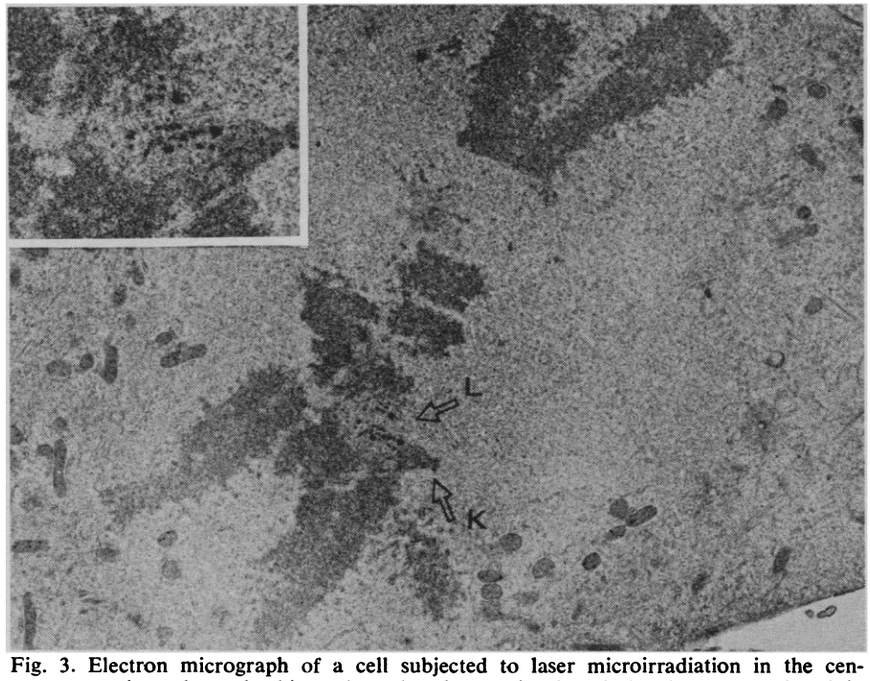


Fig. 3. Electron micrograph of a cell subjected to laser microirradiation in the centromere region where the kinetochore has been missed and the damage produced in chromatin near the kinetochore. The kinetochore appears to be pulled out of the chromosome (*K*, kinetochore; *L*, lesion material). The inset shows high magnification of the kinetochore region.

Cloning Procedure

With the ability to delete selected chromosome regions and entire chromosomes, it became necessary to develop a reliable cloning procedure for single cells. The procedure developed is outlined in Fig. 4. The target cell is irradiated in a Rose chamber containing several thousand cells. After irradiation, the cell is observed, photographed, and followed by the time-lapse videotape system (*11*) until mitosis is complete and the cell has reformed a nuclear membrane. A small ink circle is drawn around the cell on the outer surface of the Rose chamber cover glass. The culture chamber is next placed on an inverted microscope inside a sterile laminar flow hood and the irradiated cell relocated. The Rose chamber is opened, and the top cover glass (the cover slip not containing the cells) is carefully removed. By means of a micromanipulator mounted on the microscope stage, the cells adjacent to and up to 2 millimeters around the irradiated cell are dissected away. The chamber is reassembled and subjected to several washes with fresh culture medium in order to remove any floating cells. After this step, the chamber is placed back under the videotape microscope and continually taped for 48 hours. Subsequent divisions are recorded and carefully noted. The continual videotaping permits identification of any cells that migrate into the vicinity of the irradiated cell. These cells are killed by intensive laser irradiation. Once the irradiated cell begins to divide and form a clone, the culture medium is changed every third day. When approximately 200 to 300 cells are in the clone, the chamber is opened, and a sterile metal cylinder is placed around it. The bottom rim of the cylinder is coated with sterile stopcock grease so that a watertight seal is formed with the cover glass. One to two milliliters of 0.125 percent trypsin is placed in the cylinder over the cells. After 5 to 30 minutes, the free clonal cells are drawn into a syringe and transferred to a plastic T_{30} flask with 20 ml of culture medium containing 20 percent fetal calf serum. After 24 hours the medium is changed, and subsequently it is changed weekly. When the transferred clonal cells form a monolayer in the flask, they are further subcultured, frozen, and subjected to karyotypic analysis.

The weakest point in the cloning procedure is the transfer of cells from the Rose chamber to the T_{30} flask. There are risks of (i) too much trypsin killing a large proportion of the cells, (ii) microbial contamination, and (iii) nonclonal cells being transferred. A series of cloning experiments was therefore conducted in order to perfect the methodologies. With the nucleolus being used as a genetic marker, cells were cloned that had two nucleoli instead of one. This was possible because the PTK_2 cells normally have one nucleolar organizer on their X chromosome. However, there is always a small proportion of cells (1 to 3 percent) that have two nucleoli. It was theorized that these cells (i) contained an extra X chromosome, (ii) were tetraploid, or (iii) had an extra functional nucleolar organizer that was normally repressed. If the extra nucleolar condition was heritable, then it would be possible to use this characteristic as a marker in perfecting the cloning procedure. Indeed, it was possible to clone selectively the two nucleolar cells and establish viable populations. The karyotypic analysis demonstrated that these cells were tetraploid. Several relatively stable tetraploid cell lines have been established by this procedure (Fig. 5). The establishment of these clonal tetraploid lines not only demonstrates the reliability of the cloning procedure, but also provides a good system for training personnel.

Cloning Laser Irradiated Cells

The data summarizing the cloning experiments are presented in Table 1. Both nonirradiated cells and cells ir-

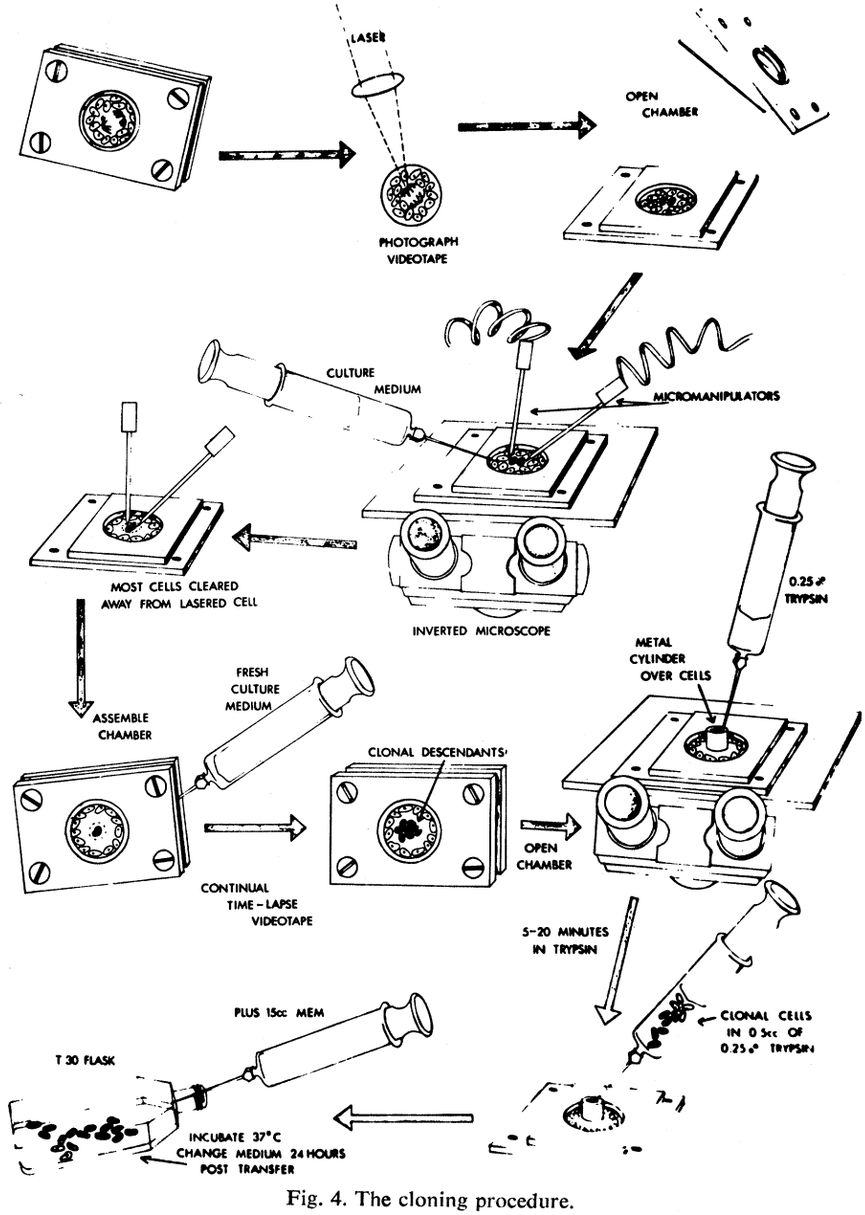


Fig. 4. The cloning procedure.

radiated in a nonchromosome area were used as controls. A total of 102 cells were microirradiated in a preselected area of chromosome No. 1 and were successfully carried through the micromanipulation and microdissection phases of the cloning procedure. Thirty-seven of these cells underwent at least one additional mitosis, and 21 of the 37 went through at least two additional mitoses. Nine of these cells were successfully cloned into viable populations. Of these nine clonal sublines, five were derived from cells that had a partial DNA deletion on the long arm of chromosome No. 1, and four were from cells that had either an entire or a major portion of chromosome No. 1 deleted by centromere irradiation. Two of these four were from the normal near-diploid PTK_2 cell line, and the other two were from one of the stable tetraploid PTK_2 cell lines. It was thought that for the deletion of whole chromosomes, better cloning success would be attained if chromosomes were selectively removed from a tetraploid rather than from a near-diploid cell line. With respect to chromosome No. 1, this reasoning was fallacious because it proved possible to clone equally well the cells of both lines. The overall success (9 percent) of cloning cells that had had a chromosome region irradiated was significantly less than the success of cloning the two control groups of cells (Table 1). However, 9 percent was higher than one might normally expect considering the amount of radiation exposure, the damage inflicted on the DNA, and the stress of the cloning procedure on the cells.

The karyotype analyses of the cell lines subjected to partial chromosome deletion showed that these cells were no different from the parental cell lines or from each other as far as total number of chromosomes was concerned (Fig. 6). The modal total chromosome number for the five clonal cell lines was 14, and the modal large chromosome number was 8 (there were three No. 1 chromosomes, two each of Nos. 2 and 3, and one X chromosome). These results indicate that the laser damage to a small region (0.25 to 1 μm) of chromosome No. 1 does not result in the permanent loss or visible alteration of a chromosome No. 1 from the cell line. However, this result does not preclude the possibility that the actual chromosome that was irradiated was not replicated but, rather, an addi-

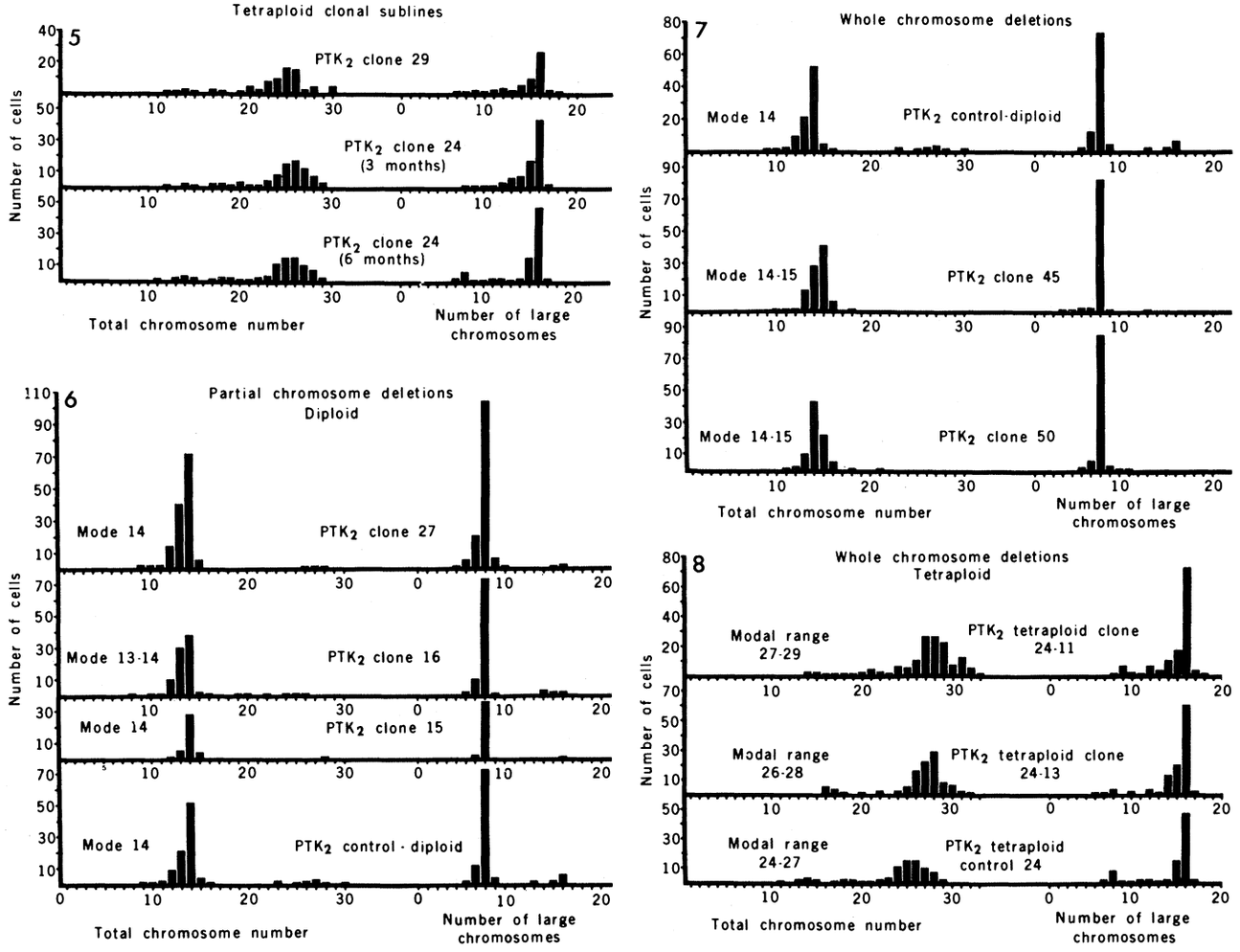


Fig. 5 (top). Karyotype distribution of tetraploid clonal sublines of PTK_2 cells established by means of the cloning procedure illustrated in Fig. 4. The number of nucleoli was used as a marker. Fig. 6 (bottom). Karyotype distributions of three of the five cell populations derived from single cells in which a 0.5-μm region of chromosome No. 1 had a DNA deletion made with the laser microbeam. Lower distribution is of the nonirradiated control population. Fig. 7 (top). Karyotype distribution of cells in which a whole chromosome No. 1 was deleted from the parental cells. Both clones 45 and 50 appear to have replaced the lost large chromosome and, in addition, each appears to have an extra small chromosome in a large proportion of cells. Fig. 8. Karyotypes of tetraploid clonal sublines in which a large No. 1 chromosome is deleted from the parental cells.

tional chromosome No. 1 was produced either by an abnormal mitotic event or by the double replication of a nonirradiated chromosome No. 1. The results also do not eliminate the possibility that the damaged DNA component of the laser irradiated chromosome remains altered in all the subsequent daughter cells. Unless that segment of DNA were genetically expressed, detection of the mutation would be impossible. Ultrastructural and cytochemical analysis of the chromosomes in these cell lines will help resolve this question.

The situation with the whole chromosome deletion experiments is more complex. Both clonal lines derived from the near-diploid parental cells lost a whole, or a major part of, chromosome No. 1 as an immediate consequence of irradiation. However, when the karyotypes were determined (Fig. 7) (approximately 2 to 3 months later), both lines had the normal number of large chromosomes, 8. Most of these cells had three of chromosome No. 1. However, the total chromosome number of these two cell lines varied significantly from the parental line. One line, designated PTK_2-C45, had a modal chromosome number of 15, and the other, designated PTK_2-C50, had a modal number of 14 with a significantly high number of cells with 15 chromosomes. In the parental population, only 4 percent had 15 chromosomes, whereas with the clonal line PTK_2-C50, 26 percent of the cells had 15 chromosomes. It appears that in both of these clonal cell lines there is a significant percentage of cells with an extra small chromosome. The occurrence of this extra chromosome could be due to the production of a chromosome fragment as a result of the kinetochore being missed by the microbeam during irradiation. This fragment would thus be the kinetochore and some chromatin material capable of undergoing replication.

The restitution of the normal number of chromosome No. 1 suggests the existence of a cellular mechanism for the maintenance of a specific number of this chromosome.

The results with the tetraploid cell lines were similar to the results with the previous two cells (Fig. 8). The modal number of large chromosomes was 16 for both lines. This number is identical to that in the parental tetraploid line and is twice the number in the near-diploid parental line. The total chromosome number in the tetraploid clonal lines was significantly larger, as a result of the presence of a small fragment chromosome, than in the tetraploid parental line. Unlike the near-diploid parental line, the distribution of total chromosome number in the tetraploid lines was not sharp. No clear-cut modal number could be discerned. The most commonly occurring total chromosome numbers in the parental line were 25, 26, and 27, and in the two deletion tetraploid clonal lines, 26, 27, 28, and 27, 28, 29, respectively.

The results with the tetraploid cells also suggest the existence of a cellular mechanism for restoring a lost chromosome No. 1.

Conclusions

In this article I have presented data that indicate the feasibility of attaining the five objectives outlined in the introduction. It should be possible to assign genes to specific chromosome regions by (i) selective DNA deletion of a 0.25- to 0.5-μm segment of one or both homologous chromosomes, (ii) deletion of one or both entire homologous chromosomes, or (iii) combining cell fusion with selective deletion of whole chromosomes and then deletion of chromosome segments.

By laser microirradiation it should be possible to determine which chromosomes and chromosome regions are essential for immediate cell survival by removing from individual cells whole chromosomes, and chromosome segments from each of the chromosomes in the karyotype, and then assessing the cloning efficiency of each cell. For example, we have already determined that removal of one large chromosome No. 1 from PTK_2 cells does not prevent the cell from undergoing a subsequent mitosis.

It should also be possible to generate new classes of mutants by damaging small selected areas of DNA with the laser beam and then cloning the irradiated cells—but this has yet to be demonstrated. This procedure might reveal recessive alleles on the nonirradiated homolog, or might result in the direct production of a genetic mutation. Irradiation of identical places on both homologous chromosomes could result in deletion of a genetic locus which ultimately might be detected as a deficiency in a metabolic pathway or some other cellular abnormality.

Studies on chromosome stability and DNA constancy can be conducted with laser irradiated cells. For example, the karyotypic analysis of chromosome No. 1 suggests that a cellular mechanism exists to maintain the constancy of this chromosome in both the diploid and tetraploid cell lines. The same approach could be used with each of the chromosomes in the karyotype. Various cytochemical procedures could be used for making quantitative DNA studies of the cells, and chromosome and DNA analyses could be performed at varying times following laser microirradiation.

It might also be possible to study the repair of chromosomal damage caused by laser irradiation. The cells could be examined by autoradiographic, cytochemical, and electron microscopy procedures at varying times after irradiation, and because the precise location, time, and nature of the mutational event would be known, subsequent analysis of repair and alteration would be facilitated.

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