animals have been studied thus far, with the duration of pregnancy at time of cesarean delivery of placentas ranging from 27 to 170 days. In every instance particles could be located within 30 minutes while scanning thin sections at a magnification of × 20,000. Although the preparations varied in quantity and ease of recognition of particles, all yielded several typical budding and complete structures.

Sites of budding particles were usually confined to the "inner" surface of the syncytiotrophoblast, that is, the side opposite the villus surface (Fig. 1). Most frequently, buds appeared to emerge from cytoplasmic processes formed by convoluted plasma membranes at the junction between syncytial and cytotrophoblast layers (Figs. 2 to 4). Where cytotrophoblast cells were no longer present, budding occurred from the podocytic processes of the syncytiotrophoblast into the basal lamina. Occasionally, buds were observed in intracytoplasmic cisternae (Figs. 5 and 6). Average particle diameter was determined to be 100 nm; both complete and budding forms usually possessed electron-lucent cores of approximately 50 nm surrounded by three layers. Albeit superficial, description of these structures as "C type" was further based on the absence of surface spikes, eccentric nucleoids, and intracytoplasmic, 70-nm "A-type" particles. Therefore, the criteria for classification as a C-type virus as described previously (*5*) have been met.

These observations of C-type structures in "normal" primate placentas at various stages of pregnancy would appear to contribute substantial support to current views on viral etiology of neoplasia and differentiation. Furthermore, it is most intriguing that these structures are so prevalent in trophoblast tissue, which is both highly invasive and immunologically unique. Obviously, caution must be exercised in interpreting these observations, particularly because of the polymorphic nature of the ultrastructural aspects of the trophoblast. Chandra *et al.* (*6*) have reported the finding of C-type virus particles in normal human embryonic muscle and liver tissue. This observation would tend to support the findings reported here on the presence of C-type particles in the placenta, another fetal tissue. From more extensive electron-microscopic studies of other primate, especially human, placentas and embryonic tissues, we have evidence of the presence of these particles in rhesus monkey and human placentas. In vitro studies are necessary to isolate and grow these putative viral agents for characterization with respect to group-specific antigens, RNA-directed DNA polymerase, and other criteria.

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Cloning of Rat Kangaroo (PTK_2) Cells Following Laser Microirradiation of Selected Mitotic Chromosomes

Abstract. *Mitotic chromosomes of rat kangaroo cells were irradiated with a green argon laser microbeam without prior dye sensitization. Deoxyribonucleic acid–negative chromosome paling was observed. The irradiated cells were isolated and cloned into viable populations.*

The deletion of chromosome segments (0.5 to 2 μm in length) by focusing a coherent laser microbeam onto specific mitotic chromosomes of tissue culture cells could be a powerful genetic mapping tool. This technique is already being used to determine the precise location of the nucleolar (ribosomal) genes of the salamander (*Taricha*) (*1*) and the marsupial (*Potorous*) (*2*). In these studies, location of the rDNA (the DNA that is complementary to ribosomal RNA) relies upon the formation of a nucleolus in late telophase and early G1 phase of the irradiated postmitotic cell.

The more general application of this technique to somatic cell genetics requires cloning and eventual establishment of cell populations from single irradiated cells. In a previous report it was demonstrated that laser microirradiated cells of the rat kangaroo (*Potorous tridactylis*, PTK_1 cell line) were capable of undergoing at least one postlaser mitosis (*3*). These cells had one of their chromosomes irradiated with the laser microbeam after 5 minutes of incubation in the vital dye acridine orange (0.001 μg/ml), which selectively sensitized the chromosomal DNA to the green laser light (*4*). Rat kangaroo cells are used in these studies because of their low chromosome number ($2n$ = 12 to 14), large chromosomes, and ability to remain flat during the entire mitotic process.

Attempts to establish that acridine orange–sensitized irradiated cells could undergo more than one additional postlaser mitosis, however, were fruitless. It was felt that perhaps the acridine orange was in some way affecting the viability of the cells, since this dye is known to be a mutagen in higher concentrations. In addition, its intercalation between the base pairs of the DNA double helix distorts the molecule. Consequently, studies were initiated to determine if DNA-negative lesions could be produced without the use of an exogenous sensitizing agent.

We now report that DNA Feulgen-negative chromosome lesions can be produced without dye sensitization by firing the laser at least six times in rapid succession (within about a 2- to 3-second period). Typical chromosome "paling" is detected within 5 seconds of the irradiation. The cells continue through mitosis and appear to have normal nuclear and cytoplasmic morphology. These cells have been isolated, cloned, and established into apparently viable populations. A description of the isolation and cloning procedure for two male rat kangaroo (PTK_2-ATCC. classification CCL56) cells follows.

These cells are designated PTK_2C567 and PTK_2C568.

Stock populations of PTK_2 cells are maintained in standard culture flasks, in minimal essential Eagle's medium (MEM) with Hanks balanced salts, penicillin and streptomycin, 10 percent fetal calf serum, and buffered with $NaHCO_3$ (0.85 g/liter). The stock cultures are determined free of mycoplasma by standard cell plating on mycoplasma agar (Gibco No. 804 AP). Twenty-four to 48 hours prior to microbeam irradiation, cells are trypsinized from stock flasks, resuspended in culture medium, and lightly seeded into Rose multipurpose culture chambers. Cells in early to middle anaphase are selected for irradiation. The argon laser microbeam is identical to the one described in numerous publications (*4*, *5*). A videotape time-lapse system attached to the microscope provides continual documentation. Cell PTK_2C567 was irradiated on the long arm of one of its larger chromosomes, and cell PTK_2C568 was irradiated on one of its smaller chromosomes. In both of these experiments we did not attempt to follow the cell from preirradiation prophase until the time of irradiation; therefore, positive identification of the irradiated chromosome was not possible. Both irradiations resulted in typical phase "paling" reactions of the irradiated region.

Subsequent isolation and cloning procedures are identical for both cells. Immediately after irradiation and photography a small circle is drawn around the irradiated cell on the outer cover slip of the Rose chamber. The chamber is removed from the microbeam system and placed on a Nikon inverted microscope that is mounted inside a sterile Edgeguard laminar flow hood. All subsequent procedures are performed with aseptic technique. The top plate of the Rose chamber is carefully removed, thus exposing the culture medium with underlying cells and cover slip. The irradiated cell is relocated through the inverted microscope and final identification is made by comparison with the videotape playback image.

After relocation of the irradiated cell, all the adjacent cells are dissected away by using a De Fon Brune micromanipulator mounted on the stage of the inverted microscope. With a finely pulled sterile glass needle (1 to 2 μm outer tip diameter), the cells in contact with the irradiated cells, and within about a 1- to 3-mm radius of them, are gently pulled way. Next, a clean sterile cover slip is placed over the gasket, and the Rose chamber is reassembled. Additional culture medium supplemented with fetal calf serum (20 percent), nonessential amino acids (0.1 m*M*), and glutamine (2 m*M*) is injected into the chamber, which is placed in an incubator at 37°C. The irradiated cell is carefully followed and photographed over a period of several days. It is usually necessary to open the chamber once or twice during this period to dissect away cells that have migrated into the vicinity of the irradiated cell population. When a population of at least 300 cells is attained, the clone is transferred to a T30 plastic flask. This is accomplished by opening the Rose chamber, removing most of the culture medium with a sterile syringe, and carefully lowering a sterile metal cylinder over the cells. The bottom of the cylinder is lightly coated with sterile stopcock grease so that it forms a good seal with the bottom cover glass of the culture chamber. One half to 1.0 ml of 0.125 percent trypsin is placed within the cylinder over the cells. The cells are continuously viewed through the inverted scope, and when free of the glass surface, they are sucked into a sterile syringe and placed in a sterile culture flask. A larger (4 ml) volume of normal MEM with *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) buffer (2.38 g/liter) is placed in the flask. Since the cells are not kept in a CO_2 incubator, HEPES buffer is needed to maintain a stable *p*H of the medium surrounding the small population of cells.

Table 1. Number of irradiated cells at various times postirradiation. After 32 days the clones were trypsinized and transferred to T30 culture flasks. At 64 days clones were growing in the flasks.

Cell identification No.	Days postirradiation				
	2	4	8	16	32
PTK_2C567	1	3	4	28	> 300
PTK_2C568	1	1	6	33	> 300

Table 1 summarizes the data for the two cells and the derived clones. It is clear from these data that cells can be cloned and established as viable populations after the deletion of DNA from a selected chromosome segment. The procedure opens the way for the directed mapping of genes to specific chromosomes and chromosome regions, and establishment of new genetic markers in somatic cell cultures.

Future work must firmly establish the parameters of irradiation and cell culture so that this rather intricate procedure will be easily repeatable and as routine as possible. In addition, careful analysis must be performed to determine the subsequent fate of the deleted chromosome region in the clonal population. Further work is needed to establish clonal populations of cells made deficient in various proportions of ribosomal (nucleolar) genes.

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Rana pipiens Complex: Hemoglobin Phenotypes of Sympatric and Allopatric Populations in Arizona

Abstract. *Electrophoretic comparison of hemoglobin samples from numerous populations of* Rana pipiens *in Arizona reveals three distinct phenotypes that closely correlate with morphological differences. Hemoglobin samples from sympatric locations contain parental phenotypes with only the occasional occurrence of a hybrid. These data support the contention that the* Rana pipiens *complex consists of several species.*

There is substantial evidence suggesting that the *Rana pipiens* complex is not one highly variable species as presently treated, but actually represents a number of primarily allopatric species. Mating calls, differences in morphology, and electrophoretic patterns all change abruptly over short