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Genetics

Direct gene transfer into human cultured cells facilitated by laser micropuncture of the cell membrane

(transformation/microbeam)

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ABSTRACT **The selective alteration of the cellular genome by laser microbeam irradiation has been extensively applied in cell biology. We report here the use of the third harmonic (355 nm) of an yttrium–aluminum garnet laser to facilitate the direct transfer of the *neo* gene into cultured human HT1080-6TG cells. The resultant transformants were selected in medium containing an aminoglycoside antibiotic, G418. Integration of the *neo* gene into individual human chromosomes and expression of the gene were demonstrated by Southern blot analyses, microcell-mediated chromosome transfer, and chromosome analyses. The stability of the integrated *neo* gene in the transformants was shown by a comparative growth assay in selective and nonselective media. Transformation and incorporation of the *neo* gene into the host genome occurred at a frequency of 8×10^{-4}–3×10^{-3}. This method appears to be 100-fold more efficient than the standard calcium phosphate-mediated method of DNA transfer.**

The introduction of exogenous genes into the cells of multicellular organisms using different techniques has proven to be a powerful approach for the study of gene regulation and function in bacteria, fungi, animal, and plant cells. Any efficient method for direct gene transfer could be of considerable value in continued progress in genetic engineering. The frequently used methods for direct gene transfer involve either chemical methods such as uptake of calcium phosphate-precipitated DNA enhanced by treatment with glycerol, dimethyl sulfoxide, or polyethylene glycol (1–3) or the manual microinjection of DNA into individual cells (4–6). DNA sequences introduced by these transformation procedures can become associated with high molecular weight DNA (7) and integrate into host chromosomes by unknown mechanisms. However, these techniques have their limitations. Although the chemical method is simple and can be performed easily, transformation frequencies are low (most protocols yield 1–5 transformants per 10^5–10^7 cells), and toxicity of the chemicals may result in cell damage. Furthermore, some cell lines are not transformed by chemical methods. The manual microinjection method yields higher transformation frequencies (1–3 in 10^3 cells) but is a very tedious technique requiring considerable skill on the part of the person performing the procedure. The microinjection method has worked very poorly in isolated protoplasts for plant transformation (8, 9). These problems might be solved by a new method for cell transformation.

Over the past 15 years the laser has steadily developed as a method to perform selective subcellular microsurgery. Numerous cell structures such as individual chromosomes, mitotic organelles, mitochondria, and the cell membrane have been selectively altered by using a variety of lasers. These alterations can be in a specific class of molecules confined to an area of less than a micrometer in diameter (10–14). In addition, interfacing a laser system with a microscope and an image array processing computer permits the exposure of single cells or individual organelles within single cells to a variety of wavelengths at various power densities. These laser microscope systems can be used to perform state-of-the-art optical and photometric examinations of the biological sample. It has been suggested that a highly focused laser beam could be used as an "optical" microbeam to produce tiny submicrometer holes in the cell membrane to facilitate uptake of exogenous DNA into cultured mouse cells (15, 16). In the present study a technique of direct gene transfer into human cells is described. We focused a 355-nm beam of a frequency-tripled neodymium–yttrium–aluminum garnet laser onto the cell membrane of human cells in culture medium containing pSV2-neo plasmid DNA. Apparently DNA entered the cell prior to the membrane alteration rehealing. Transformants were selected in media containing an amino glycoside antibiotic, G418 (Fig. 1). Southern blot analyses demonstrate the physical presence of the *neo* gene in these transformants. The integration and expression of the *neo* gene were further characterized by microcell-mediated chromosome transfer and chromosome analyses. The stability of the integrated *neo* gene in the transformants was shown by a comparative growth assay in selective and nonselective media.

MATERIALS AND METHODS

Cell Lines and Plasmid. HT1080-6TG, a hypoxanthine phosphoribosyltransferase (HPRT)-deficient human fibrosarcoma (17, 18), was used for transformation. HT1080-6TG cells were grown in minimal essential medium (MEM) supplemented with nonessential amino acids and 10% fetal calf serum. A9, a HPRT-deficient line derived from mouse L cells (19), was used as the recipient for microcell-mediated chromosome transfer. Rodent cells were maintained on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The transformants were selected and maintained in MEM alpha modification (αMEM) containing 10% fetal calf serum and G418 at the concentrations indicated. Microcell hybrids were selected and maintained in DMEM supplemented with 10% fetal calf serum, 800 μg of G418 (GIBCO) per ml, and 5 μM ouabain (Sigma).

The plasmid vector pSV2-neo containing the *neo* gene for phosphotransferase, APH (3')II derived from transposon Tn5, was described by Southern and Berg (20).

Transformation of HT1080-6TG Cells. *Laser microsurgery in cells.* Laser microsurgery was conducted on cells in Rose chambers using the third harmonic 355-nm wavelength from a short-pulsed Quantel model YG 481a neodymium–yttrium–aluminum garnet laser (10). Laser pulse duration was 10 nsec. Laser energy was controlled by a KLC model K1174

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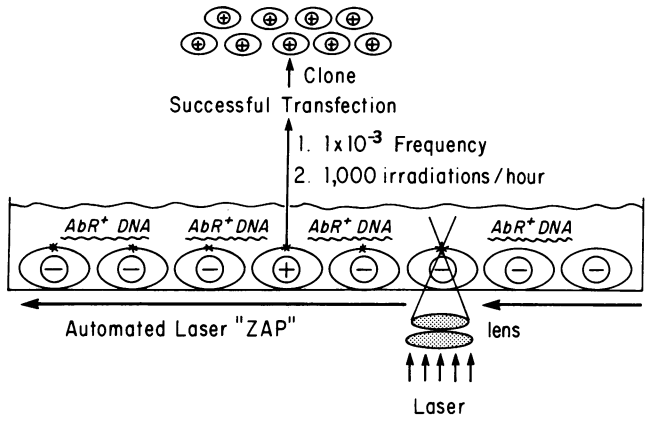

FIG. 1. Schematic representation of laser-mediated gene transfer. The cells (−) are irradiated with the focused laser beam in the presence of the plasmid DNA. The plasmid DNA, contained in the culture medium, is thought to be introduced into the cells through a very small hole momentarily made in the membrane by the laser. The transformants (+) are then selected and expanded to stable cell lines in selective medium.

continuously adjustable optical attenuator (Karl Lambrecht, Chicago) placed in the light path. Pulse energies were in the range of 23–67 μJ. All of the laser energy measurements were made with a Scientech no. 362 energy power meter at the level of the rear aperture stop of an inverted Zeiss Axiomat microscope. The laser was diverted by a series of optical mirrors and a dichroic filter into the microscope and focused to a spot of about 2.0 μm in diameter using a Zeiss 32× Ultrafluar objective. The laser beam was carefully focused onto the cell membrane of individual cells using the method described earlier (10). Single pulses were obtained by an electronic shutter synchronized with the laser. Imaging the cells and targeting the laser using the motorized microscope stage, video camera, TV monitor, and computer have been described (21). With this configuration of equipment, 1000 cells could easily be irradiated per hour.

DNA transfection and selection of transformants. The cells were seeded in standard Rose culture chambers at a density of 1×10^5 per chamber 36 hr before addition of the pSV2-neo plasmid DNA. The plasmid DNA was first sterilized and precipitated by adding sodium acetate to 0.3 M and 2 vol of ethanol. The DNA was resuspended in 1× TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 7.5). The DNA suspension was added to MEM growth medium to give a final concentration of 12 μg/ml, which was injected into the Rose chambers. The cells were incubated at 37°C for 1 hr and then subjected to laser irradiation as described above. About 6 hr after the irradiation, the Rose chamber was disassembled in a sterile laminar flow hood. The coverslip containing the cells was washed twice with MEM growth medium and transferred to a 100 × 20 mm Petri dish with 10 ml of MEM growth medium. After incubation at 37°C for 24–36 hr, the cells were trypsinized and replated into two 100-mm Petri dishes. Within 12–16 hr the medium was replaced with MEM containing G418 at a concentration of 800 μg/ml. The G418-supplemented medium was changed every 3–4 days. Independent colonies that arose were trypsinized in cloning rings and transferred to 24-multiwell plates after 14–18 days. The cells were then grown in nonselective medium for 1 day. Once established, the clones were subsequently maintained in MEM containing 600 μg of G418 per ml.

Genomic Blot Hybridization Analysis of Transformants. High molecular weight cellular DNAs were extracted and digested with individual restriction enzymes, *Eco*RI or *Xba* I. The digests were then subjected to electrophoresis in a 1.0% agarose gel, and DNA fragments in the gel were transferred to nitrocellulose filters by the method of Southern (22). After prehybridization, DNA on the filters was hybridized with ^{32}P-labeled pSV2-neo DNA at 65°C overnight. The labeling was done using the oligo-labeling method according to the procedure of Feinberg and Vogelstein (23, 24). Filters were washed, air dried, and autoradiographed (ref. 25, p. 141).

Microcell-Mediated Chromosome Transfer and Chromosome Analysis. HT1080 *neo* transformants were used as microcell donors. The microcells were prepared and introduced to recipient A9 cells as described (ref. 25, pp. 140–146) except that the concentration of colcemid was 0.02 μg/ml and that the selection medium was DMEM with 800 μg of G418 per ml and 5 μM ouabain.

The chromosome constitutions of the microcell hybrids were analyzed by using the alkaline Giemsa staining method (26). The single human chromosome in the mouse background was identified by this technique. At least 12 metaphase spreads were analyzed for each microcell hybrid.

Stability Analysis of the Transformants. The analyses were performed as described (27). Briefly, each transformant was switched to growth in nonselective medium for at least 42 days. Every 6 days, 1500 cells were plated in duplicate 25-cm^2 culture flasks; one flask contained selective medium (600 μg of G418 per ml) and the other contained nonselective medium. After 11–12 days the resulting colonies were stained and counted. Stability was calculated as the ratio of colonies formed in selective medium versus nonselective medium.

RESULTS

Cell Transformation and Selection of G418-Resistant Cell Lines. The transformation experiments were initiated to determine whether the transfer of DNA contained in the culture medium into human cells could be mediated through self-healing holes in the cell membranes produced by laser and to establish the efficiency of laser-mediated DNA transformation.

The experimental system chosen for these studies was the transfer of the *neo* gene into human fibrosarcoma cells, HT1080-6TG. HT1080-6TG cells were cultured in Rose chambers and irradiated in the presence of pSV2-neo plasmid DNA using a neodymium–yttrium–aluminum garnet laser at 355-nm wavelength as described in *Materials and Methods*. The colonies were detected and isolated in αMEM containing 800 μg of G418 per ml after 14–18 days. Fig. 2 shows the morphologies of dead cells from the control experiments and of three representative independent colonies from the transformation experiments. HT1080-6TG cells were transformed to G418 resistance at a relatively high frequency with pSV2-neo DNA using this method (on average, a frequency of about 1.6 transformants in 10^3 treated cells). This is in contrast to the lower frequencies of G418-resistant transformation using the calcium phosphate precipitation technique (about 2 transformants in 10^5 treated cells). The transformation frequencies using the laser method are in the range of $0.8–3.0 \times 10^{-3}$ based on a number of separate transformation experiments (Table 1). Control experiments (nontreated cells, cells irradiated without DNA or treated with DNA without laser irradiation) have not yielded G418-resistant colonies under these conditions. The sensitivity of HT1080-6TG cells to G418 was tested by plating cells at low cell density in multiwell plates in αMEM supplemented with various concentrations of G418. We found that 100% cell killing could be achieved at concentrations of G418 as low as 400 μg/ml (unpublished data).

Genomic Analysis of the Transformants. Molecular analyses for the presence of the transforming *neo* gene were carried out on five G418-resistant colonies as well as on control cells. High molecular weight DNA from each trans-

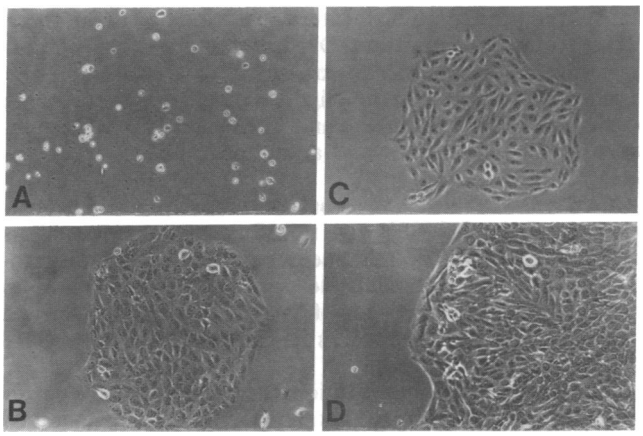

FIG. 2. Microscopic photographs of nontreated and transformed HT1080-6TG cells growing on the αMEM containing 800 μg of G418 per ml. (*A*) Control (nontreated cells). No resistant colonies developed under the experimental conditions. (*B* and *C*) Two independent G418-resistant colonies. (*D*) A large resistant colony after 15 days on the selective medium. (×100.)

formant was digested with *Eco*RI or *Xba* I, electrophoresed in an agarose gel, transferred to nitrocellulose filters, and probed with ^{32}P-labeled pSV2-neo DNA (Fig. 3). The results revealed that the untreated HT1080-6TG cells do not contain the pSV2-neo plasmid-related sequences; however, cell DNA obtained from G418-resistant transformants contained DNA sequences homologous to the pSV2-neo DNA. Moreover, the hybridization patterns of DNA isolated from individual transformants were not identical. This indicates that each independent transformant has its own organization of the integrated pSV2-neo DNA sequences with probable variation in the locations of integration and gene copy number among these transformants. Since the pSV2-neo plasmid contains a single *Eco*RI restriction site and no *Xba* I site, each integrated plasmid copy should produce two hybridizing fragments when digested with *Eco*RI but only one hybridizing fragment when digested with *Xba* I if there is no rearrangement of the plasmid DNA (20). From the number of hybridizing fragments on the autoradiogram, we infer that transformants 1:C4 and dS each contain a single copy of pSV2-neo plasmid DNA. Transformants 3, 2, and 7 appear to contain two copies of pSV2-neo plasmid; however, the hybridization pattern of DNA obtained from transformant 3 differs from that expected, which may be due to modification of the input pSV2-neo DNA during transformation. Expression of the *neo* gene in pSV2-neo-transformed cells can be inferred from the fact that all of the transformants tested are resistant to a high concentration of G418 (900 μg/ml).

Table 1. Transformation of HT1080 cells with pSV2-neo plasmid DNA

Cone of pSV2-neo DNA, μg/ml	Irradiated cells, no.	Isolated and clonal G418-resistant colonies, no.	Transformation frequency
0	0	0	—
12	0	0	—
0	1250	0	—
12	1250	1	8×10^{-4}
12	1250	1	8×10^{-4}
12	1000	2	2×10^{-3}
12	1000	3	3×10^{-3}
12	1000	2	2×10^{-3}
12	1000	1	1×10^{-3}

Transformation frequency is expressed as the fraction of cells irradiated in the presence of DNA that produces viable colonies in selective medium that have been isolated and expanded. All colonies were further characterized by Southern blot analyses to confirm that they were truly independent clones.

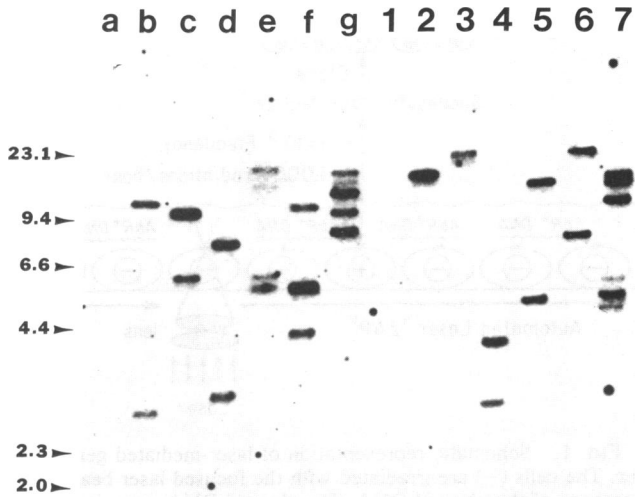

FIG. 3. Southern blot analysis of laser-mediated G418-resistant transformants. High molecular weight DNA was isolated from nontreated HT1080-6TG cells and from representative G418-resistant cell lines that had been transformed with pSV2-neo DNA and digested with either restriction endonuclease *Eco*RI or *Xba* I. After separation on a 1% agarose gel, DNA was transferred onto a nitrocellulose filter and hybridized with ^{32}P-labeled pSV2-neo DNA. Lanes a–g, DNA restricted with *Eco*RI; lanes 1–7, DNA restricted with *Xba* I. Lanes a and 1, DNA isolated from nontreated HT1080-6TG cells (negative control); lanes b and 2, c and 3, d and 4, e and 5, and f and 6, DNA isolated from transformants 1:C4, dS, 3, 2, and 7, respectively; lanes g and 7, DNA isolated from cells that had been shown to contain multiple integrated pSV2-neo DNA sequences previously (positive control). There is a single recognition site in pSV2-neo DNA for *Eco*RI cleavage and no recognition site in pSV2-neo DNA for *Xba* I cleavage. The numbers beside the arrowheads refer to the molecular sizes of DNA in kilobase pairs, and marker molecular sizes were derived from *Hin*dIII DNA restriction fragments in an adjacent lane on the gel.

Microcell Hybridization and Chromosome Analysis. Although the genomic blot hybridization data suggest that exogenous pSV2-neo DNA was integrated into host chromosomes, it was still possible that the exogenous neo DNA somehow rearranged and replicated independently as an extrachromosomal unit (7). To confirm that the exogenous pSV2-neo DNA in the transformants was actually integrated into chromosomes, the two transformants shown to contain a single copy of pSV2-neo plasmid DNA, dS and 1:C4, were used as microcell donors. Microcells were prepared as described in *Materials and Methods* and fused to mouse A9 cells. Microcell hybrids were selected on DMEM containing 800 μg of G418 per ml and 5 μM ouabain, and the colonies were isolated and expanded. The presence of human chromosomes in these hybrids was examined by using the alkaline Giemsa differential staining technique: the human chromosomes stain blue, whereas mouse chromosomes stain magenta with blue centromeric regions. The single lighter-staining human chromosome (Fig. 4, arrow) was observed in

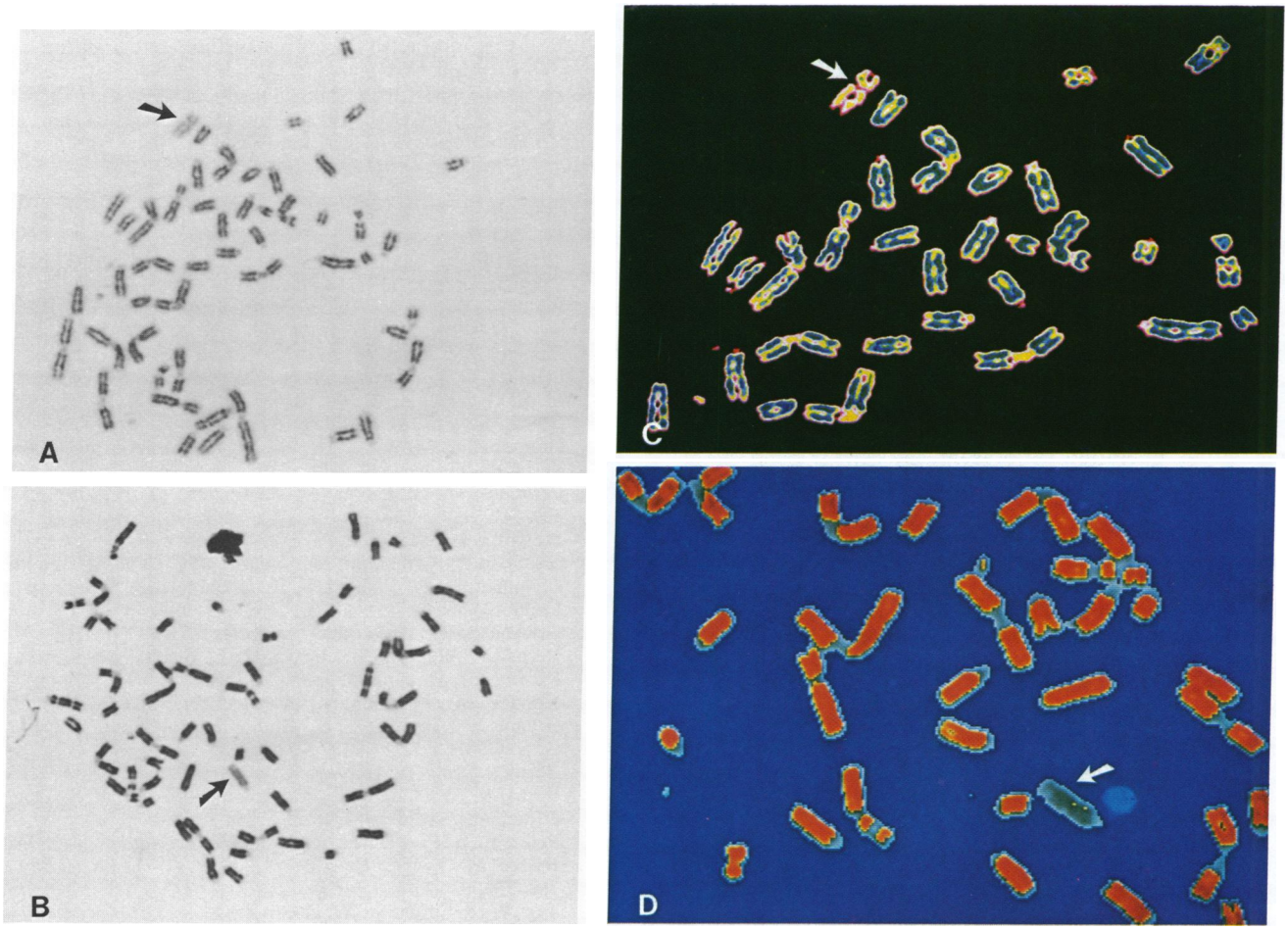

FIG. 4. Alkaline Giemsa-stained metaphase spreads of human–mouse microcell hybrids containing single human chromosomes (arrow). (*A*) Hybrid 1:C4/A9. (*B*) Hybrid dS/A9. Each hybrid represents a mouse A9 fusion to a different human transformant containing singly integrated pSV2-neo DNA. (*C* and *D*) Computer enhancement of *A* and *B*. (×1050 for *A* and *B*.)

each microcell hybrid derived from transformants 1:C4 (Fig. 4*A*) and dS (Fig. 4*B*), respectively. A computer enhancement (Fig. 4 *C* and *D*) of the mitotic spreads facilitates identification of the human chromosome. These results demonstrate that the pSV2-neo plasmid DNA has indeed integrated into host chromosomes. From these data alone, we cannot determine the identities of the human chromosomes. However, it appears that the human chromosome in hybrid dS/A9 belongs to the D group, whereas the human chromosome in hybrid 1:C4/A9 belongs to the C group, judging by the chromosomal morphologies.

Stability of Transformants. The stability of the transferred G418-resistant phenotype was analyzed by growing each transformant on nonselective medium for at least 42 days and then plating an aliquot of each cell line in selective medium and nonselective medium at various times. The number of colonies arising in both media was scored and the ratios were plotted versus the time in nonselective medium. Most of the transformants tested were found to be stable, showing no loss of the resistant phenotype. The results obtained from three representative independent transformants were shown in Fig. 5.

During the course of the experiments, we found that the ratios obtained from a couple of transformants did vary (data not shown). However, after being grown in nonselective medium for 42 days, these cells when replated at 1/12th the cell density and challenged in selective medium still remained fully resistant to a high concentration of G418 (900 μg/ml). Thus, these transformants also appear to be stable.

DISCUSSION

We have developed an efficient method to transfer plasmid DNA into human fibrosarcoma HT1080-6TG cells. Our data demonstrate that a short pulsed laser of 355 nm can be focused on the surface of cells, thus facilitating the uptake of

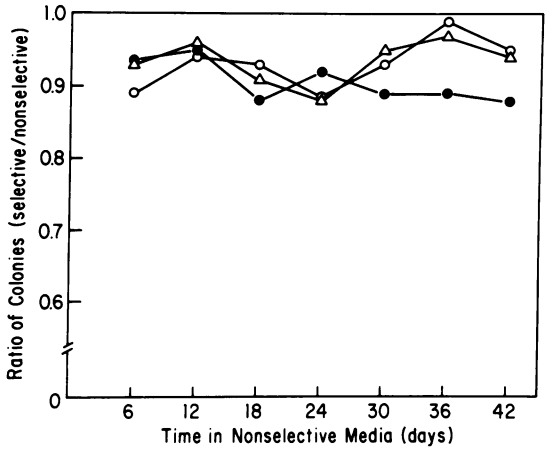

FIG. 5. Stability data for G418-resistant transformants when grown in nonselective medium. The number of colonies formed in αMEM containing G418 versus nonselective medium is taken as a measure of the fraction of cells retaining the G418-resistant phenotype.

plasmid DNA from the medium. The transferred *neo* gene was incorporated into host chromosomes and expressed. Most of the transformants that have been examined appear to contain the nonrearranged introduced *neo* gene and have a low plasmid copy number, generally one to two copies per cell. Transformant 3 is exceptional in that restrictions with either *Eco*RI or *Xba* I produce two bands and the sizes of the fragments produced by digestion with *Xba* I are substantially smaller than that of pSV2-neo plasmid (5.7 kilobase pairs in size). Rearrangement or deletions of the introduced DNA molecules may explain this result. The majority of the transformants is shown to stably maintain the transferred phenotype for at least 42 days without selective pressure. This method is nearly 100-fold more efficient than the standard calcium phosphate technique. Even though the transformation frequency ($0.8–3 \times 10^{-3}$) is comparable with that of microinjection, the laser method has the advantage of simplicity of operation. At present, the gene transfer experiments have been conducted with the ultrasophisticated laser system described; however, this by no means implies that this hardware is absolutely necessary for transformation. A more simplistic type of laser interfaced with a standard microscope and TV system will be expected to allow one to perform gene transfer experiments using this method. Thus, when compared to the microinjection technique, the number of cells treated in a given time by an "unskilled" operator will be much greater. In addition, the actual transformation frequency achieved using this method may be higher than 3×10^{-3} since there is no evidence indicated that we have reached the optimum transformation frequency. Many factors need to be examined that could affect the transformation frequency, such as cell viability after laser treatment and the percentage of successful cell-surface "perforations." Furthermore, it is likely that laser beam intensity and wavelength can be readily modulated to achieve the optimum frequency for transformation and to accommodate various cell types, such as plant cells whose walls cannot be easily penetrated by the microinjection capillary pipette.

Although the experiments presented here have been carried out with HT1080-6TG cells, theoretically there is no reason to expect that this system should not work with other given cell types, animal or plant. This method could be of considerable value in genetic engineering, especially in plants. Direct gene transfer to isolated protoplasts using the calcium phosphate method has been reported to be a successful method in many cases (28–30); however, it requires protoplasts, and the regeneration of whole plants from isolated protoplasts is still a severe problem for many monocots. Transformation experiments with the Ti plasmid of *Agrobacterium tumefaciens* (31–33) are restricted to dicots due to the natural host range of *A. tumefaciens*. Taken together, new techniques for plant transformation are absolutely needed. The laser method might be a candidate since it does not require protoplasts and circumvents the problem of host range. Furthermore, it might be possible to facilitate gene transfer directly into pollen. Another application of this method might be its use in transforming cell types that are too fragile or sensitive to chemical techniques, such as the keratinocytes. These cells cannot be transformed with the calcium phosphate method due to initiation of terminal differentiation in the presence of calcium.

The mechanism of action of laser-mediated DNA transfer is not yet completely understood; the suggested cell-surface perforation by the laser with DNA entry into the cell followed by rapid self-sealing of the hole can only be hypothesized at this time. Determination of the structural and physiological nature of this interaction requires further investigation.

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