Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2726–2730, April 1991
Genetics

Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles

(transfection, animal/gene transfer/microprojectile bombardment/"biolistic")

R. SANDERS WILLIAMS*†, STEPHEN A. JOHNSTON†‡§, MARK RIEDY‡, MICHAEL J. DEVIT†‡, SANDRA G. MCELLIGOTT¶, AND JOHN C. SANFORD‖

‡139 Biological Sciences, Duke University, Durham, NC 27706; ‖Department of Horticultural Sciences, Cornell University, Geneva, NY 14456; ¶E. I. DuPont de Nemours Co., Medical Products, Newark, DE 19714; and *Department of Medicine, Duke University, Durham, NC 27710

Communicated by Giuseppe Attardi, January 4, 1991 (received for review July 26, 1990)

ABSTRACT **Foreign genes were expressed in liver and skin cells of live mice by using a new apparatus to accelerate DNA-coated microprojectiles into tissues. After introduction of a plasmid in which the firefly luciferase gene was controlled by the human β-actin promoter, luciferase activity was detectable for up to 14 days in mouse tissues (skin and liver). *In situ* hybridization histochemistry revealed that microprojectiles penetrated through multiple cell layers without evidence of tissue injury and that 10–20% of the cells in the bombarded area expressed the foreign gene. An advantage of the new design is that internal organs, such as liver, can be transfected without subjecting the tissue to a vacuum. This procedure potentially is applicable to a wide variety of tissues and cell types for studies of transcriptional control elements and for expression of foreign proteins in intact animals.**

Most protocols for transfer of foreign genes into somatic cells of animals involve removing the target cells from the host, propagating and transforming these cells *in vitro*, and then reimplanting the cells in the same or a different host. An alternative strategy is to introduce the foreign DNA directly into the target tissue or organs in the living animal—i.e., transformation *in situ*. A direct approach could be simpler and less demanding from a technical standpoint. Second, it may allow expression of foreign genes in a wider variety of cell types, including terminally differentiated, nondividing cells.

There have been several previous reports of successful *in situ* transformation. Both infectious (1, 2) and noninfectious (3, 4) vectors have been used to transform liver and spleen (5), tumor cells (6), neuronal cells (7), T lymphocytes (8), and muscle cells (9). The procedures for introducing the transforming material have ranged from simple injection of DNA (1, 9) to administration of DNA via specially designed vesicles (6, 10). However, to date no technique has emerged that is highly efficient, reproducible, and applicable to a wide range of target cells and tissues.

We have investigated the possibility of adapting the microprojectile bombardment process (11, 12) to the transformation of animal tissue *in situ*. This technology involves propulsion of DNA-coated microprojectiles into target cells. The commercially available device (Biolistic PDS-1000; Du Pont) uses a gunpowder discharge to impart momentum to the projectiles. The cells are placed in a vacuum chamber during bombardment in order to minimize air impedance of particle flight. This technology was first used to transform onion cells in culture (13). Subsequently, it has been employed to transform other plants (reviewed in ref. 12), microbes (14), and animal cells in culture (ref. 15; S.A.J. and R.S.W., unpublished results), as well as mitochondria (17) and chloroplasts (18). We report here the use of a new device for microprojectile bombardment to introduce and express foreign genes in intact tissues of the living mouse.

MATERIALS AND METHODS

Apparatus Design. The essential features of the new device are depicted in Fig. 1. The principles of operation are basically the same as those described by Sanford *et al.* (11). The important modifications are (*i*) the device uses high-pressure helium gas rather than a gunpowder discharge; (*ii*) the macrocarrier, which carries the microprojectiles, is a disc rather than a plastic cylinder; and (*iii*) the device is configured to be hand-held, like a wand, and can be used either with or without a vacuum at the target interface. These features contrast with those of the apparatus currently available, which requires the sample to be placed in a vacuum chamber. The use of helium gas permits more precise regulation of particle velocity; the disc permits a more even distribution of the particles; the hand-held design facilitates direction of the particle beam to internal organs; the shorter flight path allows the technique to be applied to fragile tissues that may be damaged by exposure to vacuum. In addition, the device is arranged so that the gas discharge that impels the microprojectiles is deflected away from the tissue, minimizing damage resulting from a shock wave. It also differs from the modification described by Christou *et al.* (19), which uses a high-voltage electrical discharge to vaporize water droplets as the source of expanding gas.

The details of operation of the device are illustrated in Fig. 1. A small cylindrical chamber (0.8 × 2.5 cm) is pressurized with helium. The gas is restrained by a stack of Kapton (DuPont) discs (2 mils thick, 1.3 cm in diameter), using one disc per 300 psi of gas pressure (1 psi = 6.89 kPa). Electrical activation of a solenoid causes a plunger to pierce the restraining discs. The descending plunger also seals against an O-ring, blocking further escape of helium from the gas source. The macrocarrier is a separate Kapton disc (2 mils thick, 2.4 cm in diameter) that is mounted 1 cm from the restraining membrane and held in place with glycerol. The expanding gas drives the macrocarrier through a 0.8-cm flight path to a stopping screen that arrests the flying disc but permits the microprojectiles to strike the tissue, which is positioned 1–2 cm from the stopping screen. These components are contained within a steel cylinder 6 cm in diameter and 20 cm in length. This vessel receives the helium discharge after puncture of the restraining discs. For bombardment of skin, a seal was made at the distal end of the vessel and a

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†Present address: Departments of Internal Medicine and Biochemistry, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-8573.
§To whom reprint requests should be addressed at present address.

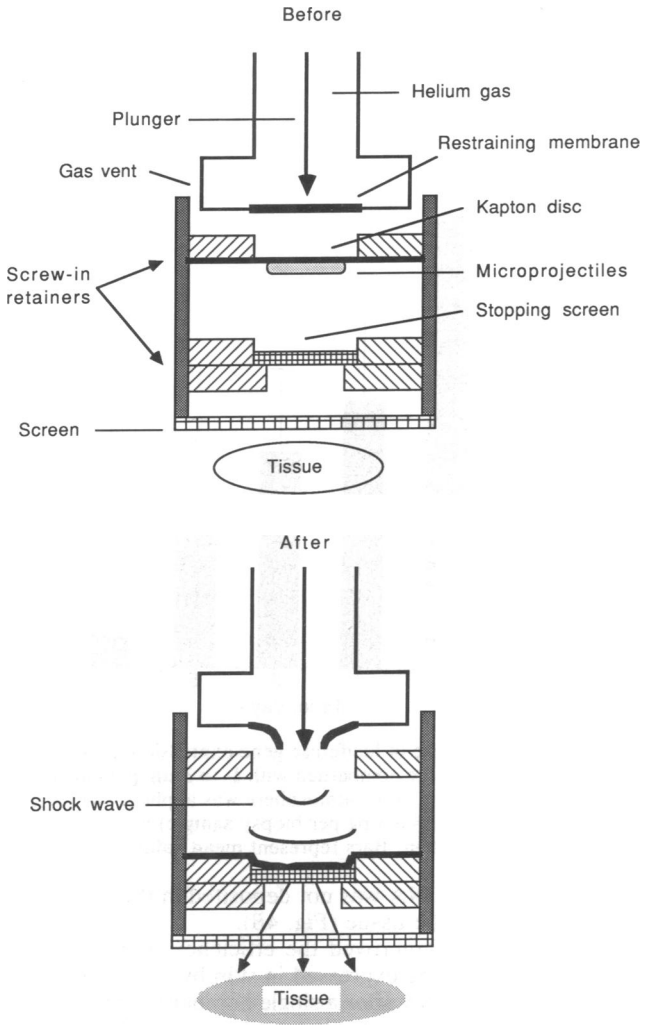

FIG. 1. Basic features of the helium-driven apparatus. DNA-coated microprojectiles are spread onto a macrocarrier disc. A membrane restraining the helium gas is ruptured by an electronically driven plunger and the disc is propelled toward the target tissue by the resulting shock wave. The macrocarrier is stopped by a screen while the microprojectiles continue on to penetrate the tissue. Further details are given in the text.

partial vacuum (0.03 or 0.3 atmosphere for hindlimb or ear, respectively; 1 atmosphere = 101.3 kPa) was drawn in the chamber below the restraining membrane. For bombardment of liver, the chamber was open to atmosphere. Most experiments were conducted at a helium pressure of 1300 psi.

Tissues were bombarded with gold particles (Alfa, Ward Hill, MA) having a range of diameters from 1 to 3 μm or from 2 to 5 μm, or with tungsten particles (Sylvania) having an average diameter of 3.9 μm. Microprojectiles were coated with DNA as described (13), by mixing sequentially 25 μl of gold or tungsten microprojectiles in an aqueous slurry, 2.5 μl of DNA (1 mg/ml), 25 μl of $CaCl_2$ (2.5 M) and 5 μl of free-base spermidine (1 M). After 10 min of incubation, the microprojectiles were pelleted and the supernatant was removed. The pellet was washed once with 70% ethanol, centrifuged, and resuspended in 25 μl of 100% ethanol. The DNA-coated microprojectiles were spread onto macrocarrier discs and the ethanol was allowed to evaporate in a desiccator before firing.

Plasmids. The foreign gene used for most analyses was the firefly luciferase coding region, isolated as a 1.9-kilobase (kb) *Bgl* II–*Bam*HI fragment from pJD207 (20). This gene was fused to a 4.3-kb genomic fragment from the human β-actin promoter (21). This promoter–reporter fusion (pHβ-LUC) was carried in pUC19, and a simian virus 40 late-region polyadenylylation signal sequence was added in a position 3′ to the luciferase gene. A second construct contained the human growth hormone coding sequence fused to the fatty acid-binding protein gene promoter (22), which is active in hepatocytes.

Assessment of Foreign Gene Expression. Luciferase activity was assayed with a Berthold 9500C luminometer by measurement of peak light emission in the presence of excess ATP and luciferin (20). The bombarded area was excised, minced, and homogenized in 0.2 ml of extraction buffer (20). Luciferase activity in tissue extracts was expressed in mass units (pg) by comparison to the activity of purified enzyme (Sigma). Background activity (<0.3 pg per biopsy sample) in tissues bombarded with plasmids without a functional luciferase gene was subtracted from results. Human growth hormone was assayed in tissue extracts and serum by radioimmunoassay (Nichols Institute Diagnostics, San Juan, CA). Tissues or plasma analyzed after mock bombardment (no DNA on microprojectiles) exhibited antibody binding similar to the blank control (<0.1 ng/ml), which was subtracted from each sample determination. Samples were compared to a standard curve prepared with known concentrations of purified human growth hormone.

The cellular distribution of luciferase mRNA in skin was assessed by *in situ* hybridization 1 day after bombardment. The tissue was frozen and cryosectioned at 10-μm thickness. The sections were dried onto gelatin/chrome alum-coated slides, fixed with 4% paraformaldehyde, and hybridized with ^{35}S-labeled synthetic oligonucleotide probes complementary to luciferase mRNA. Sections were probed simultaneously with two 35-base probes that hybridized to two different regions of the luciferase mRNA, in order to increase the signal (23).

Animals. Adult female CD-1 (Charles River Breeding Laboratories) mice (25–30 g) were used. Animals were anesthetized by injection with a mixture containing ketamine and xylazine (33 μg of each per gram of body weight) at 5 mg/ml. Hair in a 4-cm^2 region was removed with a commercial depilatory. For liver bombardment an additional injection of ketamine (22 μg/g) was usually administered during the procedure. Aseptic surgical procedures were used to expose and position the left lateral lobe through a 1-cm incision of the muscle wall of the abdominal cavity. After bombardment the lobe was repositioned, the incision sutured, and an antibiotic ointment applied. This procedure, including bombardment, was completed within 25 min and all animals survived.

RESULTS

Our initial experiments were performed using an adapter-insert for the commercially available device. This allowed a small area of the target tissue on a live mouse to be exposed to vacuum and bombarded with microprojectiles. The design and operation of this adaptation are described elsewhere (16). The adapter-insert permitted expression of luciferase in skin but was ineffective for gene transfer into liver, since the hepatic capsule could not be exposed to vacuum without injury. The introduction of foreign genes into internal organs by microprojectile bombardment required the device described in this publication. In addition, this device proved superior for gene transfer into skin.

Transfection of Skin. The skin of the hindleg was bombarded with 3.9-μm tungsten or 2- to 5-μm gold microprojectiles coated with the β-actin promoter–luciferase reporter construct. The animals were sacrificed at various intervals after bombardment, ranging from 1 to 14 days. The bombarded tissue (0.6-cm diameter) was excised and luciferase activity was determined. Bombardment with the commer-

cially available device fitted with an adapter-insert resulted in expression of luciferase activity (1000-fold over background) on day 1, and lower levels of activity remained detectable up to day 4. This transient expression probably is attributable to the fact that most of the microprojectiles come to rest in the first few layers of epidermal cells. By day 3 most of the few remaining microprojectiles were found in the dermis (data not shown). Apparently, most of the cells containing microprojectiles are cornified and sloughed as part of their normal maturation. Histological examination of the tissue revealed very little cell damage or inflammation. However, microscopic hematomas were evident in a minority of bombarded areas (data not shown).

In situ localization indicated that luciferase mRNA was expressed in 10–20% of the cells in the epidermis of the bombarded area (Fig. 2). Tissues were sectioned 1 day after bombardment and probed with radioactive oligonucleotides complementary to luciferase mRNA. Fewer than 5% of the cells expressing the foreign gene were in the dermis. No localized hybridization of the probes was evident when tissue sections were pretreated with RNase or in sections bombarded with DNA lacking a functional luciferase gene.

A similar protocol was applied to express luciferase in cells of the ear. We reasoned that the microprojectiles would more readily penetrate the thin epidermis of the ear and introduce DNA into more cells within the dermis, potentially permitting stable expression of foreign genes and improved access to the circulation for secreted gene products.

The time course of luciferase activity in the skin of the ear is depicted in Fig. 3. Expression of luciferase was detectable at high levels (4000-fold over background) in the ear and persisted for up to 10 days.

Histological examination revealed that the gold particles had penetrated as far as the cartilage in the middle of the ear (Fig. 4*A*). There was relatively little evidence of cell damage, except in regions where large aggregations of the microprojectiles had impacted the tissue. To determine whether DNA was carried deep into the tissue or removed as particles passed through the superficial cell layers, we stained the DNA with 4′,6-diamidino-2-phenylindole before precipitation onto the microprojectiles and examined tissue sections by fluorescence microscopy immediately after bombardment. Most of the deeply penetrating microprojectiles retained DNA, and DNA was not deposited in the path of the particles through the tissue (Fig. 4*B*).

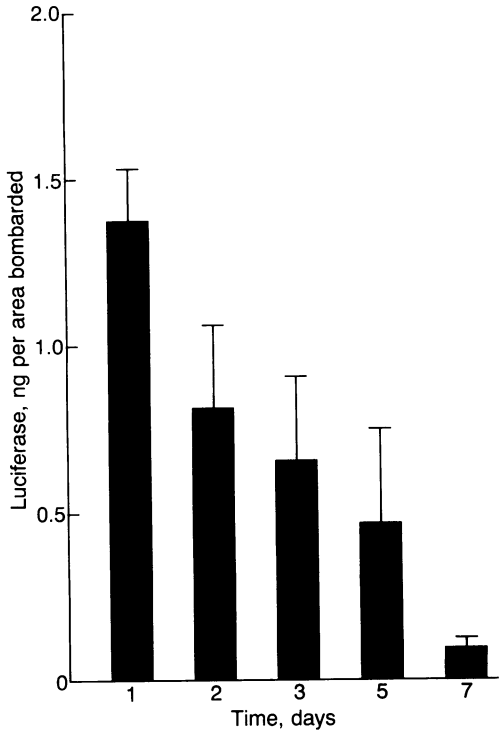

FIG. 3. Time course of luciferase gene expression in skin of the mouse ear. Tissues were bombarded with 1- to 3-μm gold microprojectiles while a vacuum of 0.3 atmosphere was applied. Values from mock bombardments (<0.3 pg per biopsy sample) were subtracted from each determination. Bars represent mean values ± SEM.

The new design improved the efficiency with which the foreign gene could be expressed in skin by severalfold (Fig. 5). Luciferase activity after a single bombardment of the ear approached 2×10^4 over background. In addition, the use of the apparatus extended the period in which luciferase expression could be detected in skin to 14 days.

Transfection of Liver. After bombardment of liver with 2- to 5-μm gold particles coated with pHβ-LUC, luciferase activity per area bombarded was comparable to that for skin

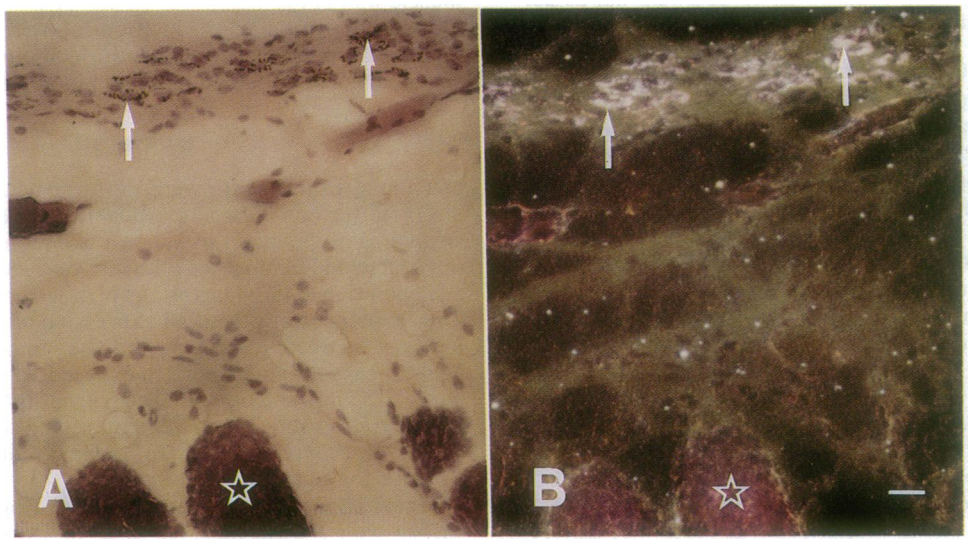

FIG. 2. *In situ* localization of luciferase mRNA in mouse skin. Bright-field (*A*) and dark-field (*B*) photomicrographs show autoradiographic labeling in the epidermal layers after hybridization to ^{35}S-labeled oligonucleotide probes. Arrows indicate positive hybridization, which appears as black grains in *A* and as white grains in *B*. The star marks a hair follicle. Skin biopsy specimens were removed and frozen 1 day after bombardment and sectioned prior to hybridization. The tissue was counterstained with hematoxylin/eosin so that nuclei appear pink. (Bar = 25 μm.)

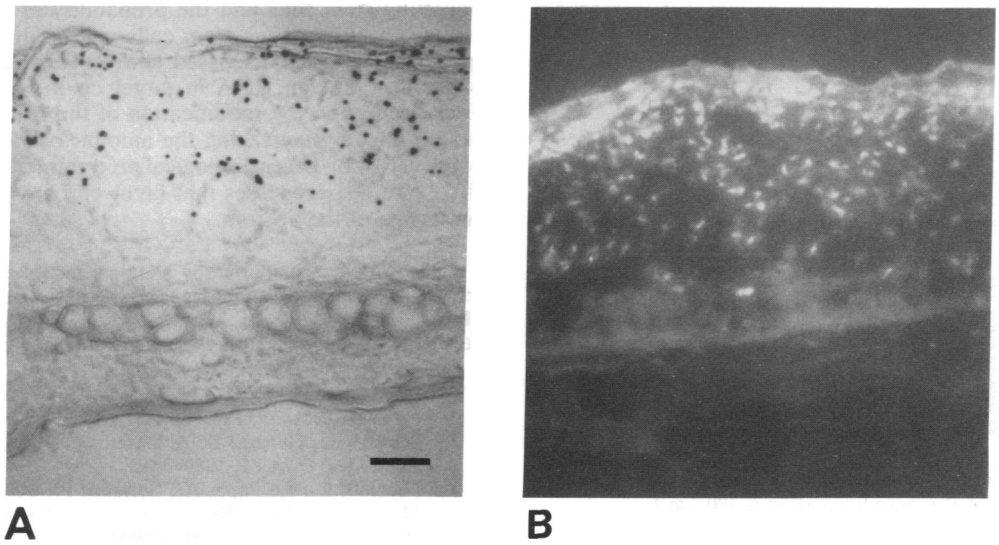

FIG. 4. (*A*) Location of the 1- to 3-μm gold microprojectiles in a cross-section of the ear from a mouse. Microprojectiles penetrated to the medial cartilage layer. (*B*) Location of 4′,6-diamidino-2-phenylindole-stained DNA immediately after bombardment. (Bar = 50 μm.)

on day 1 but declined by day 3 (Fig. 6). The microprojectiles were capable of deep penetration (Fig. 7) even though the flight path before striking the tissue was through air rather than a vacuum. The liver also was bombarded with a plasmid bearing the human growth hormone coding sequence under control of the fatty acid-binding protein promoter (22). Expression of human growth hormone (1.1 ng/ml) was detected in the blood up to 3 days after bombardment and in the excised tissue up to 23 days later. This level of activity was >10 times the detection limit of the radioimmunoassay (0.1 ng/ml).

DISCUSSION

We describe here an improved approach for introduction and expression of foreign genes in somatic cells of intact animals. Skin and liver cells were transiently transformed by microprojectile bombardment applied to the tissue *in situ*. Because the skin of the ear and hindlimb can be exposed to vacuum without significant injury, this tissue could be transformed using a simple modification of the standard PDS-1000 device (16). However, a new, helium-driven apparatus increased the efficiency of transformation of skin cells and also permitted transformation of the liver. This device is portable and easily manipulated in a surgical setting.

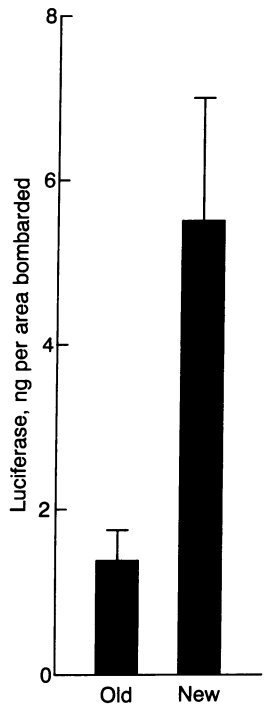

FIG. 5. Comparison of luciferase expression after bombardment using a modified PDS-1000 (old) and the helium-driven apparatus (new). The new apparatus resulted in a 4-fold increase in expression of the foreign gene in mouse skin. Values from mock bombardments (<0.3 pg per biopsy sample) were subtracted from each determination. Bars represent mean values ± SEM.

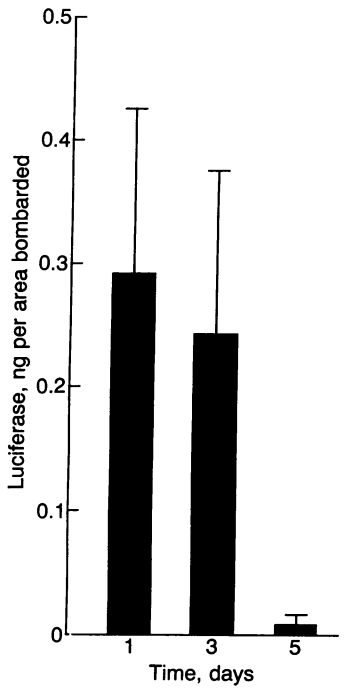

FIG. 6. Time course of luciferase gene expression in mouse liver. The livers were bombarded with 2- to 5-μm gold microprojectiles without application of a vacuum. Values from mock bombardments (<0.3 pg per biopsy sample) were subtracted from each determination. Bars represent mean values ± SEM.

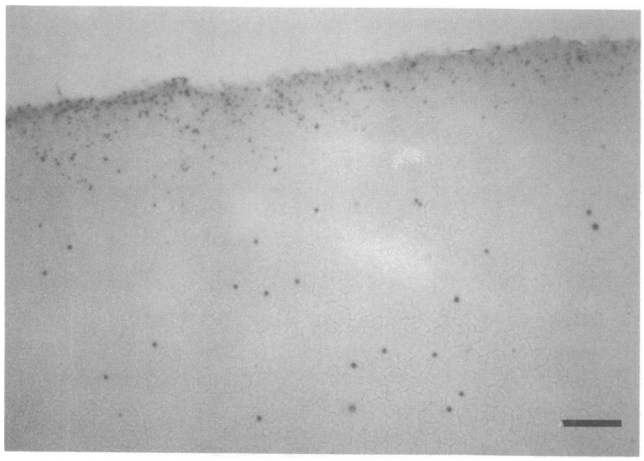

FIG. 7. Location of 2- to 5-μm gold microprojectiles in a cross-section of a bombarded mouse liver. (Bar = 100 μm.)

For this new technique to be of general use for genetic modification of somatic cells, it must meet certain requirements. Enough microprojectiles must penetrate and carry DNA in an evenly dispersed pattern into target cells with minimal damage. We have observed that the device is capable of impelling the microprojectiles through multiple cell layers of both skin and liver without significant tissue trauma in the wake of the deeply penetrating projectiles. DNA is carried along to the resting point of most of the microprojectiles (Fig. 4). A significant fraction of the cells in the penetration zone of the bombardment express the foreign gene (Fig. 2). Therefore, this approach appears to meet the physical requirements for many practical applications, especially if further refinements increase the number of cells that can be transformed with a single bombardment.

In the experiments reported here, a noninfectious, nonreplicative vector was used and only transient expression was observed. Transient expression extended for at least 14 days in skin and for up to 23 days in liver. For many potential applications, such transient expression of genes introduced into somatic cells may be adequate or desirable. For example, analyses of transcriptional control elements, expression of a circulating factor for a limited period, or expression of an antigen to elicit an immune response could be accomplished with foreign gene expression of the durations we have observed. Moreover, the duration of expression may be prolonged if nondividing cells are targeted. For example, skeletal muscle fibers *in vitro* (S.A.J. and R.S.W., unpublished results) and *in vivo* (9) appear to express foreign genes for extended periods, even when transfected with nonintegrative and nonreplicative plasmids. Other mitotically inactive cells (e.g., neurons) also may serve as useful targets.

On the other hand, many important goals of somatic-cell gene transfer, particularly those directed at correction of genetic defects in humans, probably will require chromosomal integration of the gene after introduction into the appropriate stem cells. The usefulness of the microprojectile technique for this purpose will depend on the efficiency of targeting stem cells and the probability of integration. The efficiency in targeting stem cells will be determined by the physical variables discussed above. The frequency of integration of foreign DNA in target cells potentially may be increased either by modification of the transformation protocol (e.g., by increasing the amount of DNA delivered to each cell or by pharmacological pretreatment of the tissue) or by exploiting processes that favor chromosomal integration in design of the vectors.

This work was supported by National Institutes of Health grants to S.A.J. and R.S.W.; a Duke–DuPont Collaborative Research Program grant to S.A.J., S.G.M. and R.S.W.; a grant from Du Pont to J.C.S.; and a grant from the Perot Family Foundation to R.S.W.

1. Dubensky, T. W., Campbell, B. A. & Villarreal, L. P. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7529–7533.
2. Seeger, C., Ganem, D. & Varmus, H. E. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 5849–5852.
3. Nicolau, C., Le Pape, A., Soriano, P., Fargette, F. & Juhel, M. F. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1068–1072.
4. Wu, G. Y. & Wu, C. H. (1988) *J. Biol. Chem.* **263,** 14621–14624.
5. Benvenisty, N. & Reshef, L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9551–9555.
6. Wang, C.-Y. & Huang, L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7851–7855.
7. Holt, C. E., Garlick, N. & Cornel, E. (1990) *Neuron* **4,** 203–214.
8. Desrosiers, R. C., Kamine, J., Bakken, A., Silva, D., Woychik, R. P., Sakai, D. D. & Rottman, F. M. (1985) *Mol. Cell. Biol.* **5,** 2796–2803.
9. Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A. & Felgner, P. L. (1990) *Science* **247,** 1465–1468.
10. Kaneda, Y., Iwai, K. & Uchida, T. (1989) *Science* **243,** 375–378.
11. Sanford, J. C., Klein, T. M., Wolfe, E. D. & Allen, N. (1987) *Particle Sci. Technol.* **5,** 27–37.
12. Sanford, J. C. (1988) *Trends Biotechnol.* **6,** 288–302.
13. Klein, T. M., Wolf, E., Wu, R. & Sanford, J. C. (1987) *Nature (London)* **327,** 70–73.
14. Armaleo, D., Ye, G.-N., Klein, T. M., Shank, K. B., Sanford, J. C. & Johnston, S. A. (1990) *Curr. Genet.* **17,** 97–103.
15. Zelenin, A. V., Titomirov, A. V. & Kolesnikov, V. A. (1989) *FEBS Lett.* **244,** 65–67.
16. Johnston, S. A., Williams, R. S., Riedy, M., DeVit, M. J., Sanford, J. C. & McElligott, S. (1991) *In Vitro Cell Dev. Biol.*, in press.
17. Johnston, S. A., Anziano, P. Q., Shark, K., Sanford, J. C. & Butow, R. A. (1988) *Science* **240,** 1538–1541.
18. Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B. & Sanford, J. C. (1988) *Science* **240,** 1534–1538.
19. Christou, P., McCabe, D. E. & Swain, W. F. (1988) *Plant Physiol.* **87,** 671–674.
20. DeWet, J. R., Wood, K. V., DeLuca, M., Helinski, P. R. & Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737.
21. Leavitt, J., Gunning, P., Porreca, P., Ng, S.-Y., Lin, C.-S. & Kedes, L. (1984) *Mol. Cell. Biol.* **4,** 1961–1969.
22. Sweetser, D. A., Birkenmeier, E. H., Hoppe, P. C., McKeel, D. W. & Gordon, J. I. (1988) *Genes Dev.* **2,** 1318–1332.
23. Fitzpatrick-McElligott, S., Card, J. P., Lewis, M. E. & Baldino, F. (1988) *J. Comp. Neurol.* **273,** 558–572.