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Transfer of foreign DNA into the cells of developing mouse embryos by microprojectile bombardment

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Mouse cells of developing embryos at the 2–4 cell, morula and blastocyst stages, were bombarded by high velocity tungsten microprojectiles. About 70% of developing embryos survived the bombardment. The general embryo structure did not change as a result of the bombardment. Penetration of the tungsten microparticles into the embryo cell nuclei was found at all stages being investigated, and tungsten particle localization on mitotic chromosomes was demonstrated. The total DNA of the mice born from the bombarded embryos was analyzed by dot-blot hybridization and PCR with post-hybridization. The most important results were obtained in experiments with blastocysts. In three cases of blastocyst bombardment, the presence of transferred plasmid DNA (pSV3-neo) was revealed. Transfected cells were shown to be located in the fetal membrane as well as in the embryo. The bombardment of mouse culture cells resulted in their transfection and the production of G418-resistant clones.

High velocity mechanical DNA transfer; *neo* gene; Mouse culture; Embryo cell transformation

1. INTRODUCTION

The development of the microprojectile cell bombardment technique [1–3] has greatly enhanced the possibilities of transfection of different types of cells. First suggested for the transfection and genetic transformation of plant cells [4,5], the method was later applied successfully to the genetic transformation of animal cells in culture [6]. G418-resistant mouse NIH 3T3 cells were obtained and the presence of the *neo* gene in their DNA was revealed by dot-blot analysis [6]. Later transient expression of some foreign genes in rodent tissues after high velocity DNA injection has also been described [7–9].

In our previous work [10] we used cell bombardment to introduce foreign genes (β-galactosidase and neomycin phosphotransferase) into developing fish embryos. Fertilized oocytes of Loach (*Misgurnus fossilis*), rainbow trout (*Salmo gairdneri* Rich) and zebra fish (*Brachydanio rerio*) were used in the experiments. About 70% of the embryos survived the bombardment. The activity of both transferred genes was revealed in the fish which developed from the bombarded eggs. The presence of the sequences of the neomycin phosphotranspherase gene in the total zebra fish DNA was detected by means of PCR amplification and Southern hybridization.

In the present investigation we have used cell bombardment technology to introduce a *neo* gene sequence into mouse cells of early developing mouse embryos. Since the work required PCR analysis of a small number of transfected mouse cells the methods were tested on bombarded mouse cultures.

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2. MATERIALS AND METHODS

2.1. *Mouse embryos*

Early embryos were obtained from C57/BL/GJ mice by standard techniques. Embryos at the 2–4 cells, morula and blastocyst stages were used for experiments. Immediately before bombardment the embryos were placed in a plastic dish, and covered with 2% agar, with a minimal amount of culture medium. For every shooting experiment 60–100 embryos were used. Both the 2–4 cell embryos and the morulas were then cultured in vitro up to blastocyst stage and then transplanted into pseudopregnant females. Blastocyst embryos were transplanted immediately after the bombardment, without post-cultivation. The foster mothers were killed at 16–17 days of the pregnancy and the embryos were taken for analysis. In some cases newborn mice were sacrificed and taken for analysis.

2.2. *Cell cultures*

L929 mouse cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, gentamycin (40 μg/ml) in 5% CO_2 at 37°C. Primary mouse fibroblasts were obtained from C57/black/J mouse embryos (14–15 days of development) and cultivated under the same conditions. The cells at the third passage were taken for the shooting experiments when 48 h cultures were used. The shooting technology and isolation of G418-resistant cells for subsequent analysis did not differ from that used in the experiments with NIH 3T3 cells [6].

2.3. *Plasmid DNA*

A plasmid containing the neomycin phosphotransferase gene (pSV3neo) was taken for transfection. The plasmid DNA was pre-

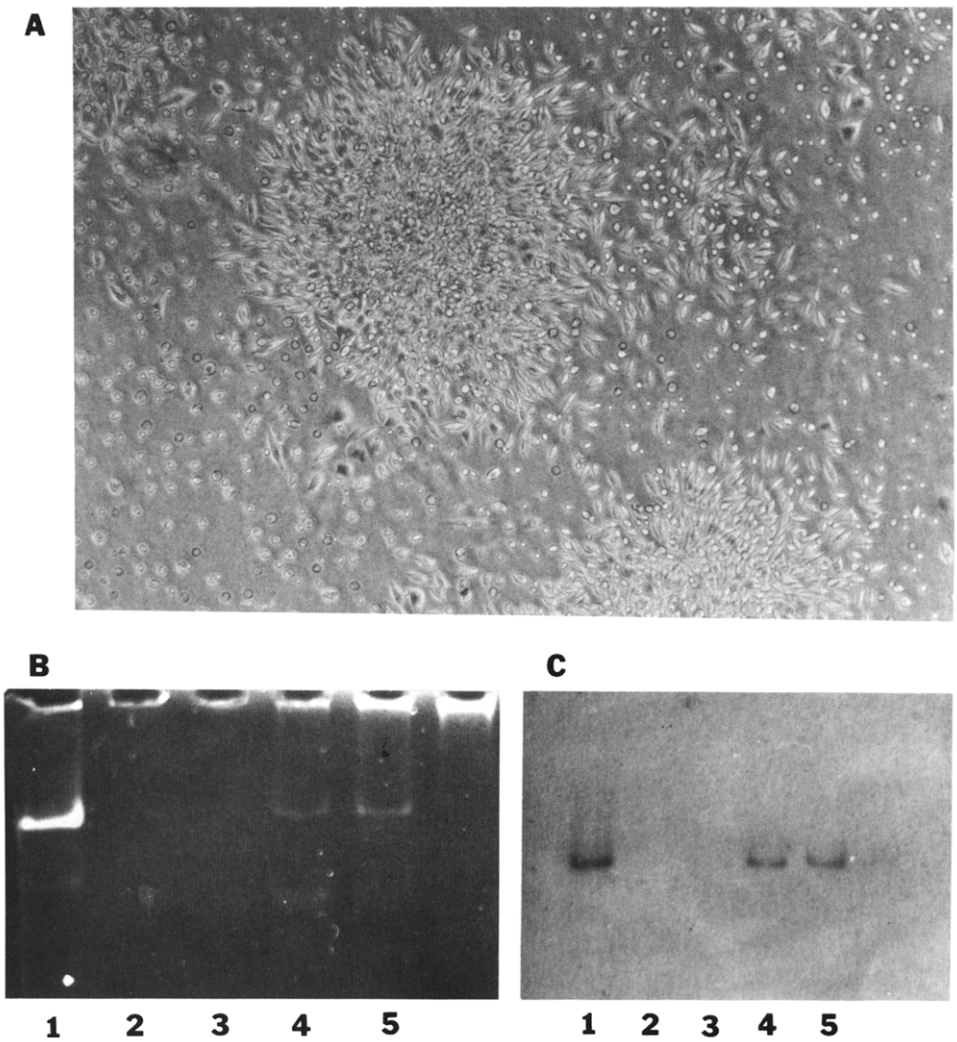

Fig. 1. (A) G418-resistant clones of L929 cell culture at the 10th day of selection. (B,C) G418-resistant clone cells DNA amplification (B) and confirmation of the PCR results by blot hybridization (C) Lane 1, positive control (plasmid DNA pSV2 neo); lanes 2,3, negative control (mouse DNA); lane 4, DNA of a G418-resistant clone (L929 cell line); lane 5, DNA of a G418-resistant clone (primary mouse fibroblasts).

pared by standard lysozyme–alkaline lysis followed by CsCl gradient centrifugation. Tungsten particles 0.3–3.0 μm in diameter were coated with plasmid DNA by calcium-phosphate precipitation as in [14–16]. Each shot consisted of 10 μl of a DNA solution (1 mg/ml) added to 10–15 mg of tungsten particles.

2.4. *Bombardment*

The shooting technology was basically identical to that used previously for the transfection of mouse cultures [6] and developing fish embryos [10]. In order to reduce damage to the delicate embryo tissues the shooting device was modified to decrease the particle speed. The distance between the end of the barrel and the eggs to be bombarded varied from 20 to 33 cm, depending on developmental stage of the embryos taken for bombardment. Maximal precautions were taken to reduce embryo contamination by dust and fulminate mercury gas.

2.5. *Morphological investigations of bombarded embryos*

Total air-dried preparations of 2-, 4- and 8-cell embryos were made according to Tarkovsky's procedure [11]. The embryos were placed in hypotonic sodium citrate solution, fixed in an ethanol–acetic acid mixture (3:1) and then stained with Giemsa (Merck).

To obtain serial semi-thin sections the blastocysts were fixed for 1–3 h in 5% glutaraldehyde in 0.1 M cocadylate buffer. They were then washed off in the same buffer and placed in a drop of molten 1.5% agar. The agar blocks obtained were imbedded in epon. Serial sections (1.5–2.0 μm thick) were stained by Toluidine blue.

2.6. *DNA amplification by PCR*

Genomic DNA was separated by a standard phenol–chloroform method and studied using amplification by the polymerase chain reaction with a slight modification, as in [10].

A group of primers for *neo* gene analysis was chosen using standard computer PCR programs. The primers were tested for possible similarity using the EMBL databank.

3. RESULTS AND DISCUSSION

Transfection of established and primary cultured cells resulted in the production of G418-resistant clones (Fig. 1A) which contained about 100 cells after 2–3 weeks of growth. The transformation efficiency varied from 1×10^{-3} to 1×10^{-5} per cell for 1 μg of circular plasmid DNA. The transformed cell clones were isolated and

taken for PCR analysis which revealed the presence of *neo* sequences in the cellular genomic DNA (Fig. 1B,C). These experiments thus confirmed previous data [6] about the applicability of high velocity DNA injection for genetic transformation of established mouse cultures, and demonstrated the possibility of using the method for the transfection of primary fibroblast cultures. These experiments also showed that the PCR technique may be used for the analysis of small amounts of DNA obtained from several hundred cells.

Investigation of air-dried embryo preparations revealed the presence of tungsten particles in the cytoplasm, in the nuclei and on mitotic chromosomes of 2–8 cell embryos and morulas. This means that tungsten particles penetrated the zona pillucida without causing visible damage and reached nuclei and individual chromosomes.

Experiments on blastocysts allowed us to study the distribution of tungsten particles in different embryo tissues (Fig. 2). Out of 22 analyzed blastocysts, microparticles were found in 20. In 12 cases the particles were located in the inner cell mass.

The direct damaging effect of the bombardment varied and depended on the stage of embryo development. Maximal damage (10%) was characteristic of the 2-cell stage and the minimal (1–2%) of blastocysts. It should be noted that the bombardment procedure had almost no effect on the subsequent cultivation in vitro and the development after transplantation to foster mothers of 2–8 cell embryos. At the same time, about 70–80% of bombarded morulas did not develop in vitro. A proportion (50–60%) of bombarded blastocysts developed after transplantation to foster mothers.

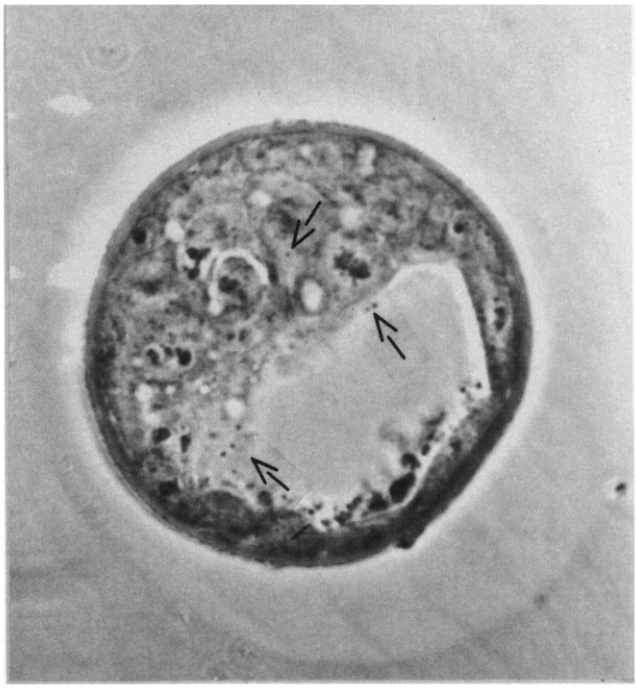

Fig. 2. A semi-thin section of a mouse blastocyst after microparticle bombardment. Stained with toluidine blue. Tungsten particles are indicated by arrows. Microphoto, 15 × 100.

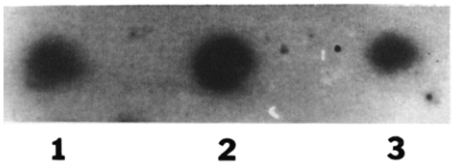

Fig. 3. Dot-blot hybridization of mouse total DNA (hybridization probe pSV2neo). Lane 1, negative result; lane 2, positive result; lane 3, negative control.

The last series of experiments were devoted to gene transfer to blastocysts. Blastocysts bombarded by microparticles carrying pSV3neo DNA were transplanted to foster mothers and their genome DNA was analyzed by PCR and hybridization.

In Fig. 3 the results of the dot-blot analysis of 16–17 day embryos are given. In one of the two embryos analyzed, a *neo* sequence in the total embryo DNA was found. The results of the dot-blot analysis were confirmed by PCR and electroblotting (the results are not given here). Fig. 4 demonstrates the localization of pSV3neo sequences both in extra-embryonic and embryonic DNA.

Our experiments have thus shown that high velocity mechanical DNA injection may be successfully used for the introduction of foreign DNA into developing mammalian embryos. It should be noted particularly that the suggested approach is unique in allowing transfer of foreign genes into embryos at the blastocyst stage, which is impossible by conventional microinjection methods. This approach thus renders possible the manipulation of cattle embryos obtained by non-surgical methods. Such experiments are currently being carried out in our group.

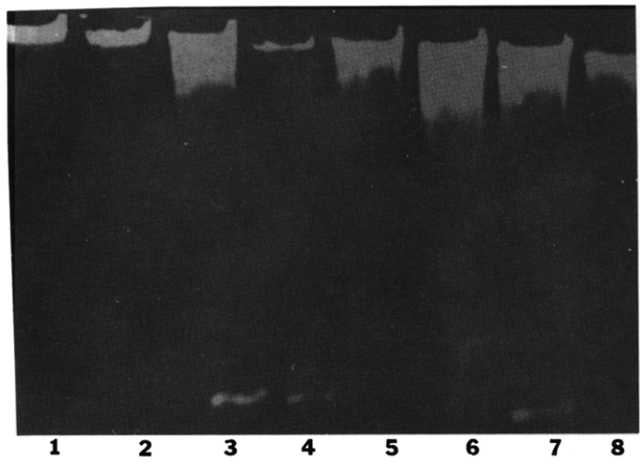

Fig. 4. Results of the amplification of embryo mouse DNA. Lanes 1,3,5,7, extra-embryonic DNA; lanes 2,4,6,8, embryonic DNA; lanes 3,4,7, positive results.

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