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Long-Term In Vivo Expression of Genes Introduced by Retrovirus-Mediated Transfer into Mammary Epithelial Cells

GILBERT H. SMITH,[1] DANIEL GALLAHAN,[1] JAMES A. ZWIEBEL,[2] SCOTT M. FREEMAN,[3] ROBERT H. BASSIN,[1] AND ROBERT CALLAHAN[1]*

Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Maryland 20892[1]; Division of Hematology, Lombardi Cancer Center, Georgetown University, Washington, D.C. 20007[2]; and Clinical Immunology Division, Department of Medicine, University of Rochester School of Medicine, Rochester, New York 14642[3]

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Nonimmortalized mouse mammary epithelial cells expressing *Escherichia coli* β-galactosidase from a murine amphotropic packaged retroviral vector were injected into the epithelium-divested mammary fat pads of syngeneic mice. Mammary glands formed from the injected mammary epithelial cells contained ductal and lobular cells, both of which expressed β-galactosidase when examined in situ more than 12 months later. These results indicate that stable recombinant gene expression can be achieved in vivo in the mammary gland without altering the growth properties of normal mammary epithelium.

A major deficiency in our understanding of the precise roles of oncogene and tumor suppressor gene activities in the process of malignant transformation results from our inability to directly evaluate perturbations in the regulation of these genes in vivo. The etiology of human breast cancer appears to involve a complex interplay of genetic, hormonal, and dietary factors superimposed upon the physiological status of the host. Mutations affecting a number of cellular proto-oncogenes and tumor suppressor genes have been implicated in the malignant transformation of breast epithelia (3). The mouse mammary gland provides an excellent experimental model within which to elucidate the functions of putative mammary oncogenes during the growth, development, and differentiation of normal mammary tissue in vivo. Any epithelial portion of the normal adult mouse mammary gland can repopulate the gland-free mammary fat pad of syngeneic mice upon transplantation (8, 12–14, 27). In previous studies, replication-defective oncogenic retroviruses (i.e., v-*myc* and v-H*ras*) have been used to infect primary mammary cells in culture (9, 28). Although no attempt was made to select for the transduced cells, when the mass-infected cultures were implanted into gland-free mammary fat pads, areas of hyperplasia were observed in some of the mammary outgrowths. However, the stability of the hyperplastic phenotype was not tested by serial transplantation of those lesions into syngeneic gland-free mammary fat pads. Clearly defined preneoplastic epithelial cell lesions have been recognized within the mouse mammary gland under a variety of experimental conditions (4, 6, 17). These partially transformed preneoplastic epithelial cell populations can be propagated indefinitely by serial passage in the gland-free fat pads of syngeneic mice. Similar repopulation of the fat pad results when dissociated normal or preneoplastic glandular epithelium is injected into fat pads or alternatively grown in culture under prescribed conditions prior to reintroduction into the fat pad (6, 17). Taken together, these observations provide an experimental basis for the development of a retrovirus expression vector system to transduce specific genes suspected of contributing to mammary tumorigenesis into primary mammary epithelial cells prior to their implantation in gland-free mammary fat pads. Paramount to the success of such a project is the long-term maintenance of transgene expression in the reconstituted mammary gland. We have performed experiments designed to (i) test the feasibility of selecting normal mouse mammary cells transduced with recombinant retroviral expression vectors before their repopulation of the mammary fat pad and (ii) demonstrate in situ the long-term expression of the transgene.

Earlier studies (4, 5) have shown that the ability of normal mammary epithelia to repopulate the fat pad is gradually lost through mitotic events in subsequent transplant generations, both in vivo and in vitro. The limited proliferative capability of normal mammary cells in culture and the apparently small number of mature epithelial cells possessing the ontologic capacity to repopulate an empty fat pad with fully functional epithelium (4, 5, 27) made selection of transduced mammary cells on the basis of an acquired drug resistance transgene an unattractive approach. Instead, the *Escherichia coli lacZ* gene was used as a reporter gene in these experiments because the product of expression, β-galactosidase, can be detected in living cells by its catalytic release of fluorescein from the fluorescein di-β-D-galactopyranoside substrate (FDG) (21), and it allows for the positive selection of *lacZ*-expressing mammary cells by cell flow cytometry. In addition, *lacZ* expression can be localized in situ by using the X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) histochemical procedure, which turns LacZ-positive cells blue (22, 24).

In each experiment, at least 10 primary cultures were infected. Mammary cells were collected after collagenase digestion of mammary glands excised from 8- to 13-week-old mice. Approximately 10^5 cells were plated onto plastic culture dishes (diameter, 60 mm) and maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and 4 mg of insulin per ml. During the first week of culture, the cells (65% confluent) were exposed to stocks of virus (10^4 to 10^5 infectious units for NIH 3T3 cells per milliliter) at multiplicities of infection (MOI) between 0.5 and 2.0 over a period of 2 to 3 h in the presence of polybrene (2.5 mg/ml). After reaching confluence the cells were transferred to irradiated feeder layers for long-term maintenance (4 to 8 weeks) (10,

* Corresponding author.

11). Duplicate cultures were analyzed for β-galactosidase expression by X-Gal histochemistry. In addition, aliquots (10^6 cells) of the combined infected population from each of the cultures were loaded with FDG substrate and analyzed in a fluorescence-activated cell sorter (FACS) for β-galactosidase enzymatic activity. Uninfected cultures or cultures infected with vector lacking the *lacZ* gene (pZIP/neo) served as controls for both of these analyses. The percentage of cells possessing fluorescent intensities at levels above that observed in nontransduced cells or those transduced with the vector alone (controls) was considered *lacZ* positive. Infected sorted and unsorted populations were injected into gland-free mammary fat pads (5×10^5 cells in 10 μl of phosphate-buffered saline). Success of repopulation of the gland was determined under a dissecting microscope after intraperitoneal injection of trypan blue into the host at 12 weeks after implantation. Our experiments were performed with retrovirus expression vectors (2, 15, 16, 18, 19, 22, 24, 26, 30) in which the *lacZ* gene is transcribed from the murine leukemia virus (MuLV) long terminal repeat (LTR). In the first experiment, primary mammary epithelial cells, after one passage in culture, were infected with the BL1 retroviral vector at an estimated MOI of 0.5 to 1.0; 20 to 30% of a sample of these cells was scored as positive for *lacZ* expression by X-Gal staining or FACS-FDG analysis. On the basis of the MOI, this number approximates the expected fraction of positive cells (1). Maintenance of these infected cells over a period of 3 months (two passages in culture) demonstrates that the fraction of *lacZ*-expressing cells was stable in ~30% of the total cell population. Introduction of the transduced mammary cells into cleared mammary fat pads resulted in mammary outgrowths in 13 of 20 pads after 3 months in vivo. Primary mammary cultures were prepared from a pool of the 13 reconstituted glands after 3 months in vivo to determine the fraction of the cell population expressing *lacZ*. We were unable to detect *lacZ* enzymatic activity by either X-Gal or FACS-FDG analysis.

The lack of transgene expression could result from either the inactivation of the transgene or the absence of the gene in these cells. To distinguish between these two possibilities, we tested DNA isolated from these cells as well as from cultures of *lacZ*-expressing mammary cells for the presence of the *lacZ* transgene (data not shown) by using the polymerase chain reaction (23). We were able to demonstrate the presence of *lacZ* coding sequences in the DNA from the cell cultures derived from the reconstituted mammary glands. However the level of signal was only equivalent to 1 *lacZ*-positive cell in 10,000 cells, as determined from experiments in which we mixed positive and negative *lacZ* packaging cells at increasing dilutions. We could not detect *lacZ* enzymatic activity in mixtures of 1:10,000 *lacZ*-positive cells by either FACS or X-Gal analysis. We conclude that the apparent absence of *lacZ*-positive cells in the reconstituted glands was probably not the result of suppression of MuLV LTR-driven *lacZ* gene expression but, rather, was due to the presence of the gene in too few repopulating cells to be detected by our enzymatic assays. One possible explanation is that the cells which are capable of repopulating the mammary fat pad in vivo represent only a minor fraction of the mammary cells which can proliferate in culture. Thus at low MOI these cells are not frequently transduced. Earlier studies (10, 11, 14, 27) indicate that mammary tissue contains only a small number of cells capable of repopulating the mammary fat pad. Therefore, to increase the probability of a successful transduction into the putative stem cells, the number of stably transduced cells must be increased in the infected population. We accomplished this increase in two ways: by increasing the MOI and by separating large numbers of infected cells away from the uninfected cells prior to their implantation in "cleared" mammary fat pads.

To approach this problem, we used the BAG retrovirus expression vector (22), which was available in a sufficiently high titer to infect primary mammary cells at an increased MOI. The *lacZ* gene in this vector, like BL1, is transcribed from the MuLV LTR. This retroviral vector also contains the neomycin resistance gene under the control of the simian virus 40 early promoter. At a MOI of 1.0 to 2.0, approximately 70% of the mammary cells exposed to the BAG virus were expressors of β-galactosidase, as determined by fluorescence activation (Fig. 1C), whereas X-Gal stained only 35 to 40% of the cells (Fig. 1A and B). A similar disparity in cells scoring positive for *lacZ* expression by FACS-FDG and by X-Gal was reported by Nolan et al. (21). They concluded that this difference was due to the greater sensitivity of the FACS-FDG technique for measuring *lacZ* enzymatic activity.

To enrich for *lacZ*-positive cells, BAG-infected cells were sorted by FACS-FDG prior to reintroduction into gland-free mammary fat pads. In the sorted population essentially 80 to 90% of the recovered cells were positive for *lacZ* activity (Fig. 1C). A proportion of the unsorted cells was directly returned to gland-free mammary fat pads by inoculation (5×10^5 cells in 10 ml per pad). The remainder was seeded on irradiated feeder layers of LA-7 rat mammary tumor cells (10, 11) and maintained for up to two additional passages. The FACS-sorted mammary cells (7×10^5 were directly recovered) were plated on 100-mm culture dishes and maintained until they reached confluency. Subsequently these cells were trypsinized, counted, and introduced into gland-free mammary fat pads. Growth was observed in three of six pads inoculated with 5×10^5 cells. In general, successful repopulation of the fat pad with a completely functional gland was observed in 50 to 60% of pads injected with 500,000 cells (Fig. 2A and B). BAG-infected cells and cells infected with the vector alone were indistinguishable in their repopulation effectiveness.

The stability of transgene expression was assessed in vitro. Six months after implantation of the unsorted *lacZ*-positive cells into the fat pads, transduced mammary outgrowths were removed and reestablished as primary cell cultures. The BAG-transduced cells still contained the *lacZ* gene, and following X-Gal staining of portions of the BAG- and pZIP/Neo-transduced cultures, only the BAG cultures contained darkly stained epithelial organoids, indicating the presence of β-galactosidase (compare Fig. 2C and 2D). Fluorescence analysis of the two cultures showed that 27% of the cells in the BAG culture expressed β-galactosidase (Fig. 2E). The percentage of *lacZ*-positive cells in the primary cultures of the reconstituted glands was smaller than the percentage (70%) of implanted transduced epithelial cells for several reasons. First, only a fraction of the epithelial cell component of the transduced mammary cell population is capable of taking part in the reformation of the mammary glandular tree in the epithelium-divested fat pad (10, 11, 27). Second, the primary cultures recovered from the repopulated fat pads contain normal nontransduced fibroblasts and endothelial cells derived from the host. Third, it is possible that some of the original *lacZ*-positive epithelial cells may have become nonexpressors in vivo.

In situ β-galactosidase expression in reconstituted glands produced by the FACS-sorted *lacZ*-transduced mammary epithelial cells was assessed. Lobuloalveolar development

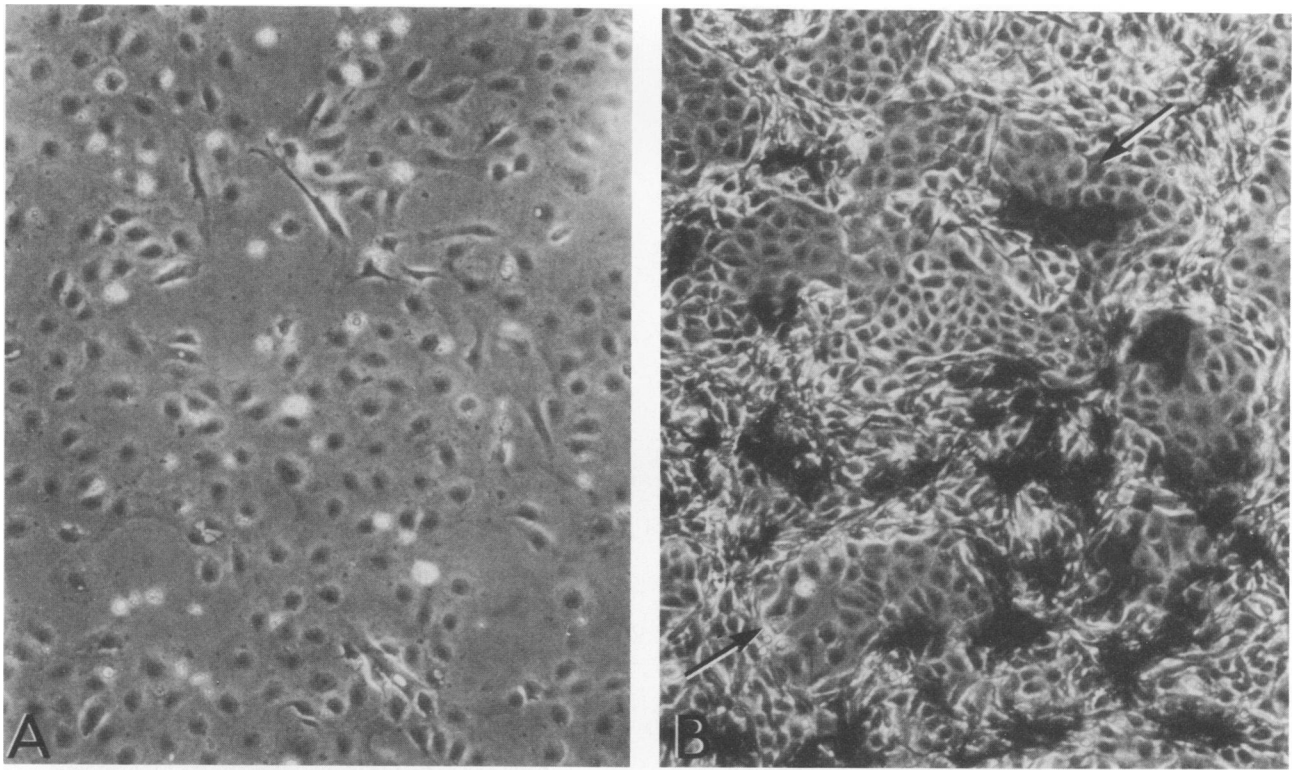
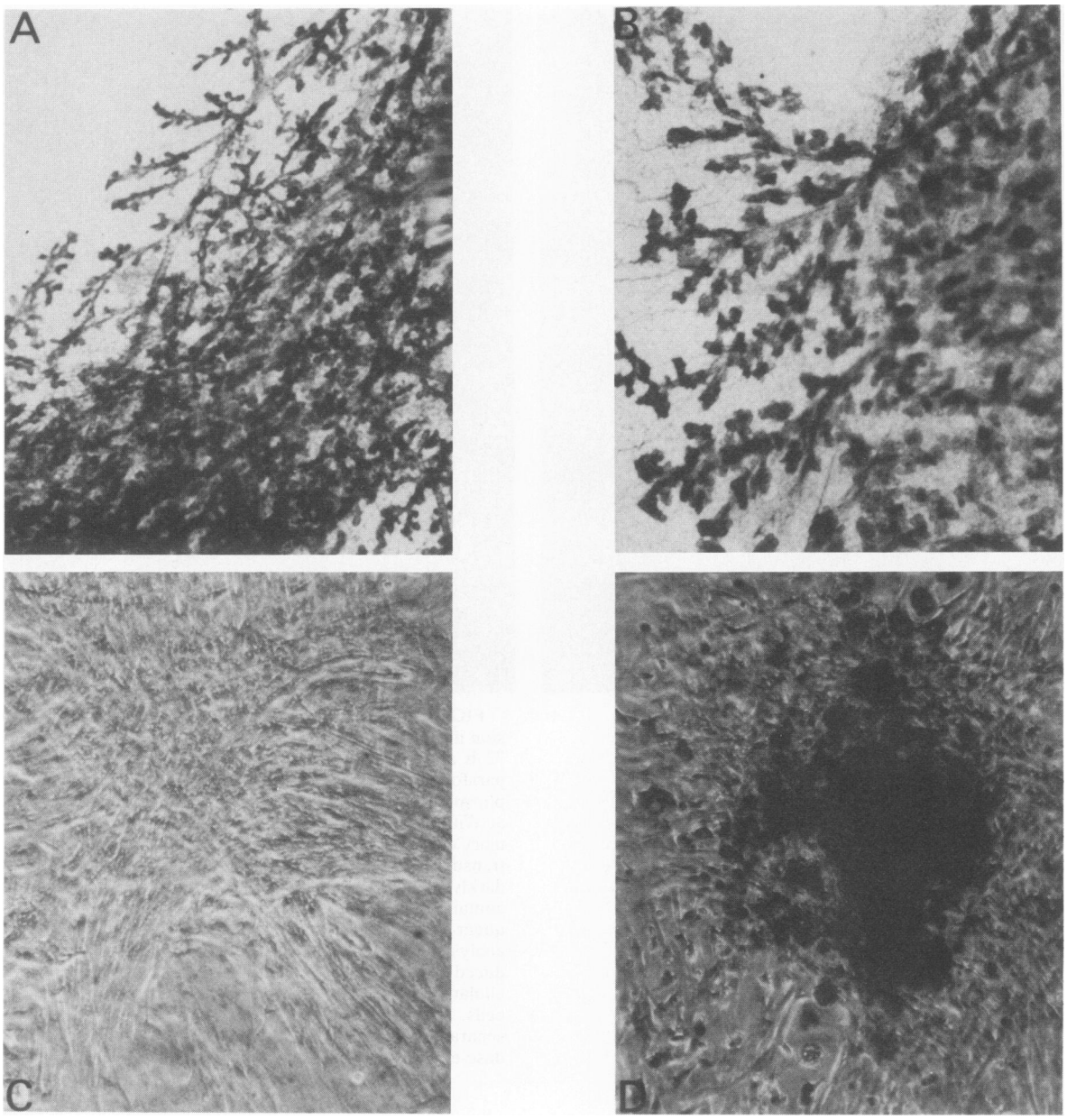


FIG. 1. Detection and quantification of *E. coli lacZ* gene expression in transduced primary mammary epithelial cell cultures. Cells, 72 h after infection with retroviral vectors, were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde for 5 min, rinsed in phosphate-buffered saline (pH 7.6), and stained for β-galactosidase activity by the X-Gal histochemical method (7). (A) Primary mammary epithelial cells transduced with the pZIP/neo vector; (B) Cells transduced with the same vector containing the *lacZ* gene. The darkly stained cells are evident; more lightly staining cells also containing β-galactosidase are indicated with arrows. (C) Subsequent subcultures of transduced cells were loaded with FDG. FACS analysis of the pZIP/neo (solid line) and BAG (dotted line)-transduced cells is shown. The *x* axis represents the strength of intracellular fluorescence, while the *y* axis represents the frequency of cells. The most highly fluorescing BAG-transduced cells were separated by FACS and cultivated. These FACS-sorted β-galactosidase-expressing cells are shown in the third peak (labelled Sorted).

and growth were stimulated by implants of estrogen-progesterone-hydrocortisone pellets (Innovative Research, Toledo, Ohio) (2). *lacZ* expression was detected by X-Gal staining of the fixed mammary whole mounts 2 to 3 weeks later (Fig. 3A and B). Whole mounts of the normal hormone-stimulated glands from the same animals were used as controls for background staining. Regardless of the method used to demonstrate *lacZ* gene activity in the reconstituted mammary glands, enzymatic activity was present in a significant proportion of the mammary epithelial cell population, even in glands which had been in place for more than 12 months. The in situ pattern of transgene expression in the reconstituted glands suggested that the patches of *lacZ*-positive epithelial cells were the result of cellular proliferation induced during the hormonal stimulation of lobuloalveolar development in the glands immediately prior to evaluation. Both glandular and myoepithelial cells stained positive for β-galactosidase enzymatic activity in these fields, suggesting that clonogenic precursors of both types of cells were positively transduced with *lacZ* in the original culture. Other regions were completely devoid of demonstrable *lacZ* activity. In our experiments, transgene-positive cells were present only in reconstituted glands in experiments in which greater than 70% of the originally injected cells were *lacZ* enzyme positive at the time of reintroduction to the mammary fat pad. Accordingly, these results demonstrate (i) that enrichment of transgene expression-positive mammary epithelial cells prior to repopulation of the gland-free mammary fat pads is important and (ii) that only a relatively small number of primary mammary epithelial cells in our cultures are clonogenic in vivo.

In this study, we have demonstrated the feasibility of repopulating gland-free mammary fat pads with genetically modified normal mammary epithelial cells in adult mice. Our results demonstrate that the transferred gene, under the control of the Moloney MuLV LTR, continues to function in



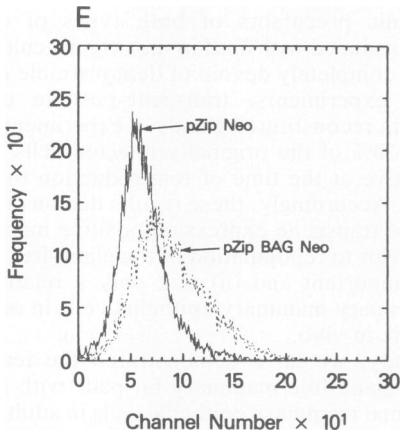


FIG. 2. Transplantation, in vivo growth, and in vitro recovery of β-galactosidase-transduced mammary epithelial cells. Aliquots of 5×10^5 cells from the mass-infected primary mammary cell cultures shown in Fig. 1 were inoculated into the epithelium-divested mammary fat pads of syngeneic mice. (A and B) Whole mounts of portions of the mammary ducts and alveoli produced by the inoculated cells transduced with pZIP/neo and BAG viruses, respectively. Six months after inoculation, the repopulated mammary fat pads were removed and treated with collagenase and primary cultures of the reconstituted glands were produced. (C and D) Portions of cultures from reconstituted mammary epithelium transduced with pZIP/neo and BAG, respectively, and stained with X-Gal. (E) Fluorescence analysis of β-galactosidase enzymatic activity in living cells from the cultures shown in panels C and D.

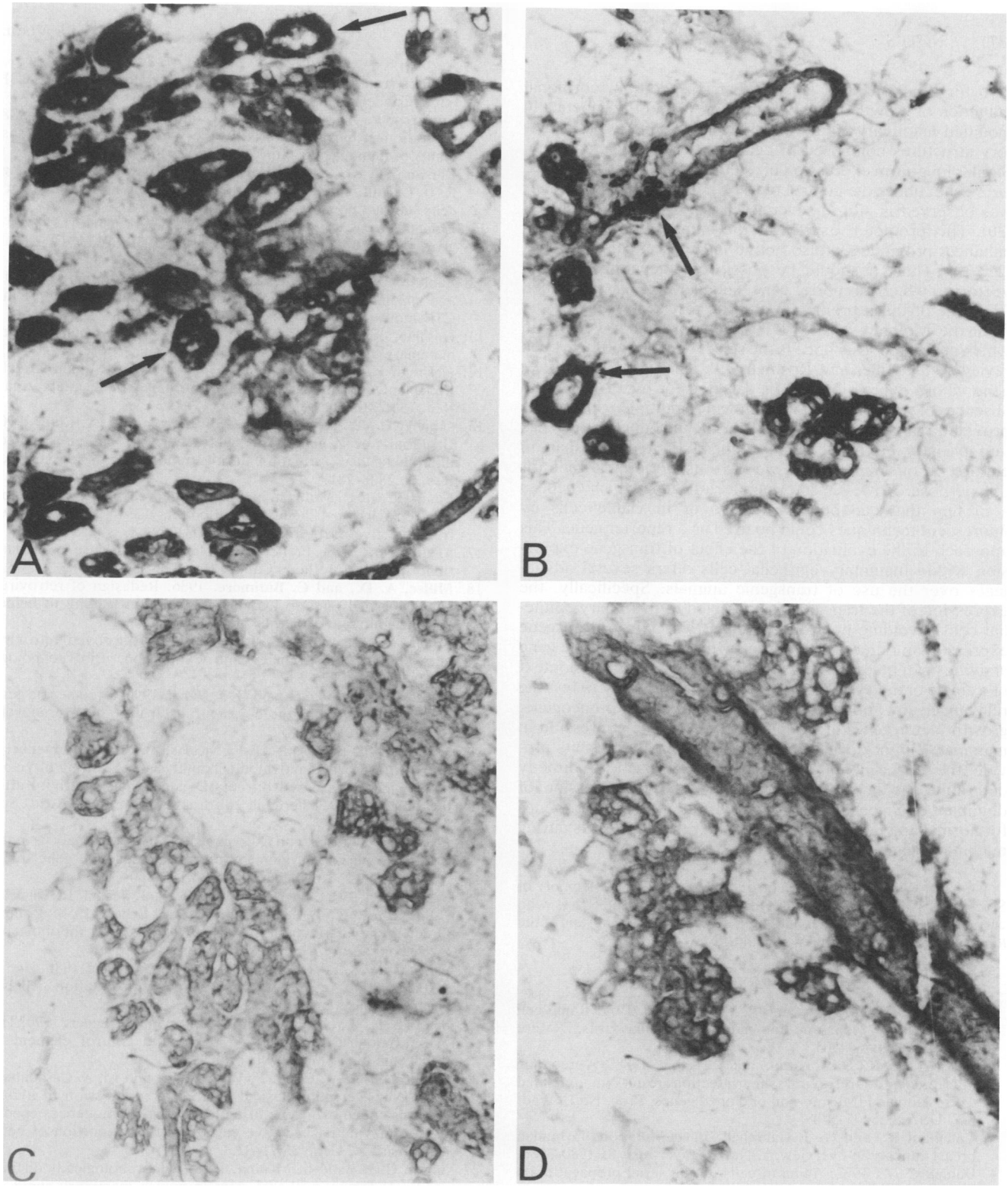


FIG. 3. Demonstration of *E. coli lacZ* transgene expression in situ. Mammary fat pads were repopulated with FACS-enriched primary normal mammary epithelial cells transduced with BAG. Prior to the removal of the tissues, females whose inguinal mammary fat pads had been repopulated 12 months earlier were implanted with slow-release hormonal pellets containing 17β-estradiol, progesterone, and hydrocortisone for three weeks to induce lobuloalveolar development. By using the procedure of Sanes et al. (21), the repopulated fat pads were fixed in 4% paraformaldehyde–0.25% glutaraldehyde in phosphate-buffered saline, pH 7.6, for 30 min and then placed in cold phosphate-buffered saline containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 for an additional 30 min and subsequently in X-Gal solution in the same buffer with detergent overnight at 37°C. Subsequently, the whole mounts were frozen and sectioned at 12 μM, dried, and counterstained with 1.0% fast green. Cells positive for β-galactosidase enzymatic activity possess a darkly staining cytoplasm. Control, intact normal axillary mammary fat pads from the same mice were treated identically. (A and B) Sections of *lacZ*-positive BAG-transduced mammary fat pads. The arrows indicate *lacZ*-positive cells present in the hormone induced lobules as well as ductal structures. (C and D) Sections of nonexperimental fat pads from the same mouse.

vivo for a period of at least 1 year. In addition, hormonal induction of growth and differentiation within the genetically modified mammary gland results in the production of secretory structures composed of cells expressing the transgene. Similar long-term expression of a retroviral vector-transduced *lacZ* gene, under the control of the chicken β-actin promoter, was observed in situ in several cell types of the vessel wall (20). This promoter, as well as the human cytomegalovirus enhancer-promoter, is also active in mouse mammary cells (25, 29). Thus, it should be possible to develop retroviral vectors in which a reporter gene, such as *lacZ*, is transcribed from one of these promoters and the test gene is expressed from the Moloney MuLV LTR. Several similar vectors in which neomycin resistance is the selectable marker have been reviewed by Miller and Rosman (19). The use of a reporter gene whose expression can be selected for by FACS in the absence of cell death and following multiple cell cycles provides a rapid means to screen for the introduction of genes into primary epithelial cells having a limited life span in culture. In our experiments we have used the *lacZ* gene for this purpose. However, in principle, any gene which encodes a protein that can be detected on or in viable cells by fluorescent techniques could be used as a reporter gene. This approach to the evaluation of the effect of transgene expression within mammary epithelial cells offers several advantages over the use of transgenic animals. Specifically, the expression of the transgene is restricted to mammary epithelial cells, avoiding possible systemic effects from transgene expression in other organ systems. In addition, multiple gene activities can be evaluated simultaneously within the context of a single organ system, providing the opportunity to test the relevance and potential cooperation of proto-oncogene, growth factor gene, or tumor suppressor gene expression in mammary tumor development. In summary, our results provide the experimental basis for an approach to directly determine the in vivo consequences of the expression of dominant oncogenes or inhibitors of tumor suppressor genes for normal and preneoplastic mammary epithelial cells during mammary gland development and differentiation.

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