EXPERIMENTAL CELL RESEARCH 207, 155–162 (1993)

β-Galactosidase Activity in Transfected Ltk⁻ Cells is Differentially Regulated in Monolayer and in Spheroid Cultures

IRENE KLÜNDER AND DIETER F. HÜLSER[1]

Abteilung Biophysik, Biologisches Institut, Universität Stuttgart, Pfaffenwaldring 57, D-70550 Stuttgart, Germany

We have investigated whether three-dimensional cultivation of cells to multicell spheroids influences the expression of a transfected gene. Ltk⁻ cells (mouse fibroblasts, thymidine kinase negative) have been transfected with a bacterial lacZ gene which was coupled to a β-actin promoter. The transfected cells synthesize β-galactosidase, a cytoplasmic enzyme which can easily be stained for histology with 5-bromo-4-chloro-3-indoxyl β-D-galactoside and for cytometry with fluorescein di-(β-D-galactopyranoside). As we have shown with monolayer cells, β-galactosidase is produced independently of cell density, medium condition, and cell cycle. In multicell spheroids, however, the portion of producing cells was reduced from ~98% to ~2% within a week. This reduction is also independent of cell density, medium condition, and cell cycle. Nonproducing multicell spheroid cells, however, regained their ability to synthesize β-galactosidase within a few days when the cells were recultivated as monolayers. Since the lacZ gene was not lost, its expression might have been regulated by its β-actin promoter. We, therefore, investigated whether the endogenous synthesis of β-actin was similarly regulated. A correlation between the distinct reduction in β-galactosidase-producing cells and filamentous or total actin concentration was not unequivocally observed.

INTRODUCTION

Adherently growing eukaryotic cells may be cultivated either two-dimensionally as monolayers or three-dimensionally as suspended multicellular aggregates—so-called multicell spheroids—where cells adhere to each other instead to an artificial substrate. With embryonic cells in three-dimensional cultures, tissue-like structural properties were reestablished and an *in vivo*-like growth and development could be observed [1]. Under the same culture conditions highly malignant human ovary and mammary carcinomas were found to form solid aggregates with histological similarities to the primary tumor [2]. Multicell spheroids, which are used as *in vitro* tumor models [for review see 3], show different growth characteristics compared to cells in monolayer culture. As was demonstrated by Nederman *et al.* [4] and Glimelius *et al.* [5] with multicell spheroids of human glioma cells, an extracellular matrix is synthesized which is similar to that of tumors *in vivo* and is reduced or even absent in monolayer cells. Components of the extracellular matrix in multicell spheroids include fibronectin, laminin, collagen, and a composition of glycosaminoglycans [6]. They are often linked via cell surface receptors (integrins) to the intracellular cytoskeleton. This explains why not only the shape but also biochemical processes of cells such as translation and processing of cellular proteins can be influenced by the extracellular matrix [7, 8].

As a major general characteristic resulting from the three-dimensional arrangement of the cells, gradients of substrates have been measured in multicell spheroids. They include pO_2 [9, 10] as well as pH [11], which decrease toward the center of the multicell spheroids as a result of anaerobic glycolysis and accumulation of catabolic products [12]. In addition gradients of glucose [13, 14, 15] and ATP [16, 17] are well documented and the probability for the existence of other gradients of nutrients, hormones, and growth factors is rather high. As a result of oxygen consumption, the inner cells in multicell spheroids become oxygen deprived, leading to the often described necrotic center which is surrounded by a rim of viable cells. Selective dissociation of these cells and their analysis with flow cytometric measurements showed that proliferating cells are localized within superficial layers whereas with increasing depth into the multicell spheroids cell cycle times are prolonged and cells remain in a nonproliferating state [18, 19]. This increase of quiescent cells toward the center presumably develops as a result of the specific micromilieu due to different gradients which are stabilized by the diffusion suppressing effect of the extracellular matrix in multicell spheroids. Similar age-dependent changes in cell cycle phase distribution could not be demonstrated in monolayer cultures. These specific conditions in multicell spheroids result in an increased cellular resistance against radiation [20, 21] and drugs

[1] To whom correspondence and reprint requests should be addressed. Fax: 0049711/6855096.

0014-4827/93 $5.00

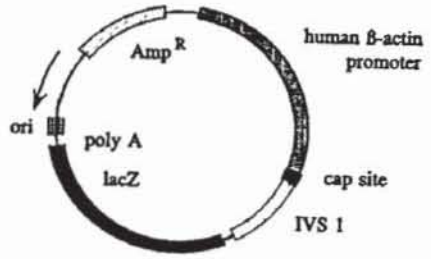


FIG. 1. Genetic map of pHβAPr-1-β-gal plasmid which was used to transfect Ltk⁻ cells.

[for review see 22] which is not observed in exponentially growing or plateau phase monolayer cells. These effects may well be attributed to a regulation of gap junctional communication between cells in multicell spheroids [23]. Furthermore, in multicell spheroids an increased protein synthesis was measured in colon carcinoma cells [24] and in rat hepatocytes [25].

Here we present evidence that even the expression of a transfected gene is only constitutive for cells grown as monolayers but not for cells cultivated as multicell spheroids. We used thymidine kinase-negative L cells (Ltk⁻) transfected with a lacZ gene connected to a β-actin promoter which expresses the cytosolic enzyme β-galactosidase. This enzyme can easily be detected by flow cytometry and by cytochemical staining in histological sections.

MATERIALS AND METHODS

Cell culture. Ltk⁻ cells, which originate from a 20-methylcholanthrene-treated primary strain of C3H mouse fibroblasts [26], were transfected by $Ca_3(PO_4)_2$ coprecipitation with an pHβAPr-1-β-gal plasmid containing an *Escherichia coli* lacZ gene connected to a human β-actin promoter (Fig. 1). Transfection and selection by treatment with geneticin was performed by Dr. H. Hauser (GBF Braunschweig, FRG). We cloned single cells by cultivating them in microtiter plates. From 70 clones, 2 with 98% producing cells were selected for our measurements. Cells were grown at 37°C and pH 7.4 in a humified incubator with an 8% CO_2/air mixture in Dulbecco's minimal essential medium (DMEM; Biochrom KG, Berlin, FRG) supplemented with 3.7 g/liter $NaHCO_3$, 100 mg/liter streptomycine sulfate, 150 mg/liter penicillin G, and 10% newborn calf serum (NCS). Cells were passaged in tissue culture flasks (Greiner & Söhne GmbH & Co. KG, Nürtingen, FRG) or petri dishes (Greiner) by treatment with 0.25% trypsin (0.38 IU/ml) in phosphate-buffered saline (PBS) without calcium and magnesium. Monolayer cultures were trypsinized, suspended in culture medium, and inoculated in petri dishes (51 mm diameter) at cell densities between 5 and 10×10^3 cells/cm². For determination of population doubling times, samples were trypsinized in daily intervals and the mean number of cells for a given time point was determined by averaging the counts obtained in a Coulter counter (Coulter Electronics Ldt., Luton, GB) from each of three dishes. Multicell spheroids were initiated by seeding single cell suspensions (3×10^6 cells per dish) in agar-coated plastic petri dishes (94 mm diameter). Coating of petri dishes (1 g agar in 50 ml PBS + 100 ml DMEM + 10% NCS) prevented the cells from adhering to the plastic surface. After a 2- to 3-day cultivation period aggregated cells were transferred into spinner flasks (Techne, Fernwald, FRG) and stirred at a rotational speed of 70 rpm in a volume of 125 ml medium. Every second day the culture medium was renewed.

Selective dissociation of multicell spheroids. For analysis of cells from different spheroid layers, 200–300 multicell spheroids were selected for diameters between 245 and 360 μm by appropriate sieving [modified after 27], transferred into a dish, and washed several times with PBS. The mean size of these multicell spheroids was determined by measuring diameters perpendicular to each other of 30 randomly chosen multicell spheroids. All spheroids were exposed to 0.25% trypsin at room temperature for 1 to 3 minutes (depending on layers) and then ice-cold PBS was added and multicell spheroids were gently pipetted. Trypsinized cells of the outer layer were dissociated from multicell spheroids and removed. After determination of the multicell spheroids' mean size without the outest layer (for 30 randomly chosen spheroids) the remaining multicell spheroids were again exposed to trypsin. This procedure was performed three to five times.

Light microscopical histology. Multicell spheroids were washed in PBS and fixed in 2% formaldehyde (v/v) + 0.2% glutaraldehyde (v/v) mixture in PBS for 2 h at 4°C. After washing with PBS several times, multicell spheroids were incubated with 5-bromo-4-chloro-3-indoxyl β-D-galactoside (X-gal staining solution: 44 m*M* X-gal, 5 m*M* potassium hexacyanoferrate(II), 5 m*M* potassium hexacyanoferrate(III), 2 m*M* $MgCl_2$ in PBS) for 24 h at 37°C. Multicell spheroids were washed, dehydrated in ethanol and in isopropanol, and embedded in Paraplast Plus (Polyscience, Washington, PA). For histology all specimens were cut to 5-μm sections and stained with hematoxylin/erythrosine (Chroma, Köngen, FRG).

Measurements of β-galactosidase activity by flow cytometry. For quantitative measurements of β-galactosidase we used fluorescein di-(β-D-galactopyranoside) (FDG) [28]. FDG is hydrolyzed by β-galactosidase to yield fluorescein which can be detected *in vivo* by flow cytometry (FACS-Analyzer, Becton-Dickinson, Mountainview, CA). The fluorescence is directly proportional to β-galactosidase activity. Monolayers or multicell spheroids were incubated with 0.25% trypsin until they could be dissociated to a single cell suspension. After washing with PBS, FDG was loaded into cells via hypotonic shock. Cell suspension and FDG solution (2 m*M* FDG in 99.5% H_2O dest./0.5% DMSO) were gently but thoroughly mixed in a 1:1 ratio and incubated for 75 s at 37°C. Loading of cells was stopped by 10-fold diluting with ice-cold isotonic propidium iodide (PI) solution (1.15 μ*M* PI in PBS) and placing the tube on ice. Keeping cells at low temperature was necessary to prevent fluorescein leakage immediately. PI diffuses only through damaged cell membranes and was used to detect dead cells. The enzymatic reaction in cells was stopped after another 4 min

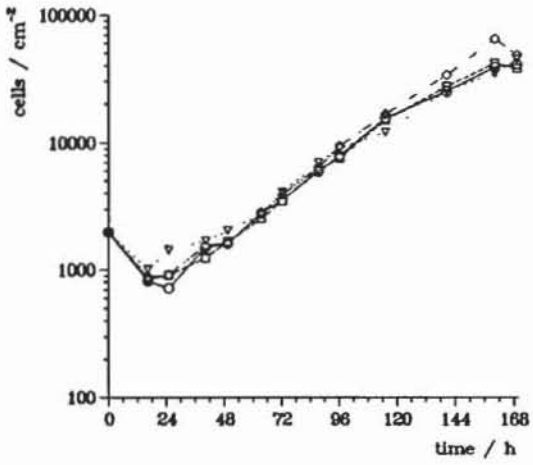


FIG. 2. Growth curves of cells isolated from four different layers in multicell spheroids and cultivated as monolayers. Layer: ○, 358–331 μm; ◇, 331–275 μm; □, 275–174 μm; ▽, 174–0 μm.

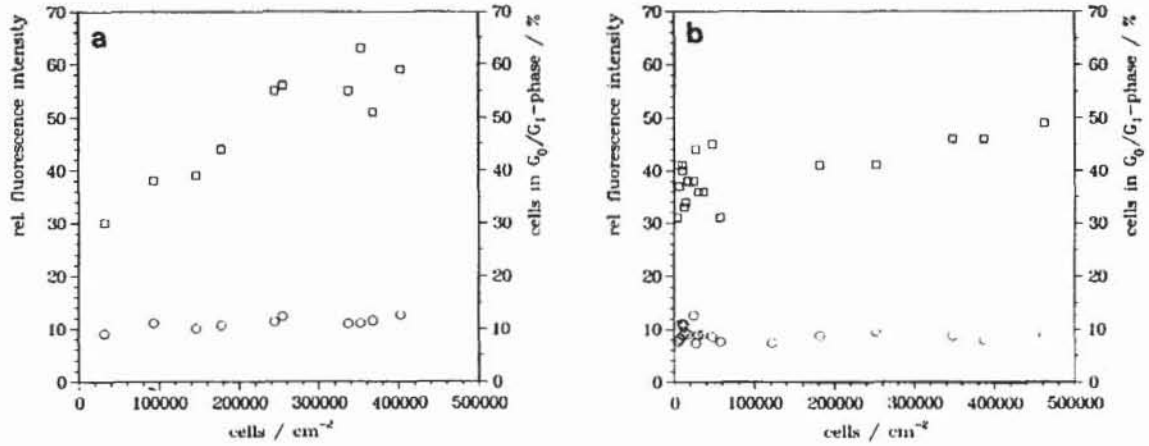


FIG. 3. Cellular β-galactosidase activity (○) and portion of Ltk⁻ cells in G_1/G_0-phase (□) in relation to cell density in monolayer culture. (a) Without renewal of medium; (b) renewal of medium every 12 h.

by adding the β-galactosidase inhibitor phenylethyl-β-D-thiogalactoside to a final concentration of 1 m*M* in PBS. FDG-stained cells had at least a 10 times higher fluorescence intensity than unstained cells which were characterized by a low autofluorescence peak which served as reference for the fluorescence peak of producing cells. Dead cells were excluded by gating in dot-blot diagramms. For each measurement 10,000 cells were analyzed and the portion of producing cells is given as the ratio of the number of producing cells to the number of living cells.

Measurements of actin concentration by flow cytometry. Fluorescein isothiocyanate–phalloidine (FITC–phalloidine) binds with high affinity to actin filaments (dissociation constant $K_D = 3 \times 10^{-7}$ [29]) and can be detected by flow cytometry. A method of Wulf *et al.* [30] was used to label intracellular filamentous actin with FITC–phalloidine. Before trypsinizing and after each step of fixation and staining, cells were washed several times with PBS. Trypsinized cells were fixed in 3.7% formaldehyde (in PBS) for 5 min and dehydrated in acetone for 4 min. Subsequently cell membranes were permeabilized with 0.1% Triton X-100 (in PBS), followed by labelling of actin filaments with 1 μ*M* FITC–phalloidine (in PBS) for 40 min. The staining procedure was carried out at room temperature.

Measurements of cell cycle phase distributions by flow cytometry. The rapid method for DNA determination without RNase digestion as described by Krishan [31] was applied to monolayer cells. Briefly, after washing with PBS, cells were trypsinized and incubated with PI staining solution (50 μg/ml PI, 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 in PBS) for 5 min at 4°C. The fluorescence intensity of nuclei was detected by flow cytometry. Cell cycle phase distributions were calculated according to the polynomial model (SFIT) which is part of the FACS-Analyzer software. The coefficient of variation was regularly between 4 and 6.

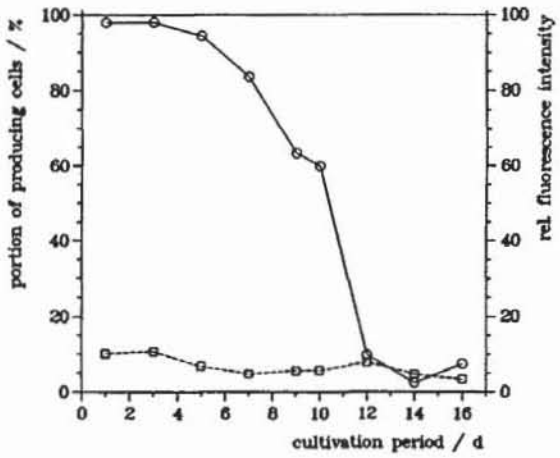


FIG. 4. Portion of producing Ltk⁻ cells (○) and cellular β-galactosidase activity (□) of remaining producing cells in relation to the cultivation period.

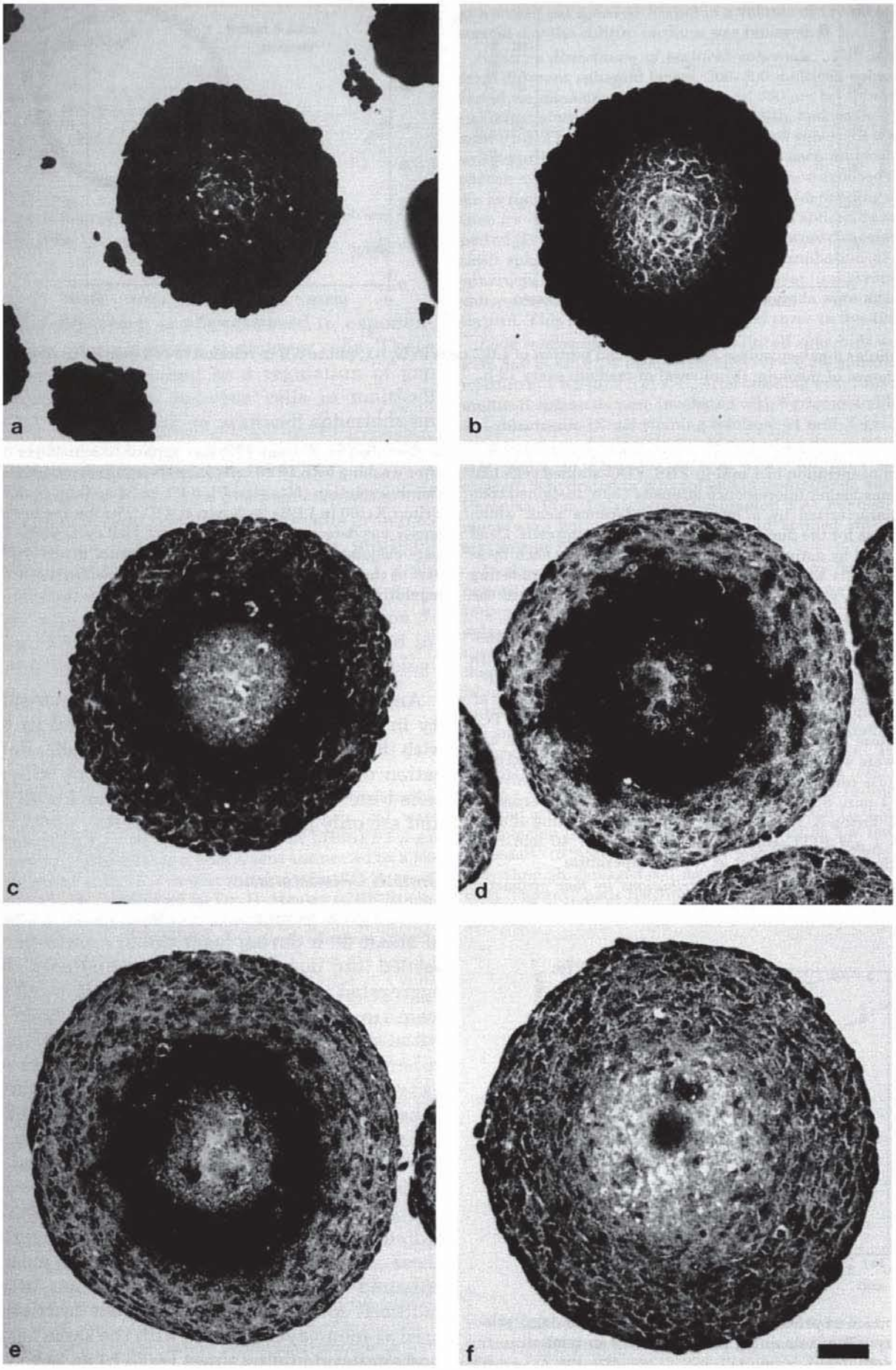
RESULTS

An age-dependent decrease of β-galactosidase activity in multicell spheroids was observed in five clones with different portions of producing cells. For quantification of the effect, clones with about 98% producing cells were used. Results were similar for all five clones and are only presented for clone A.

Growth Characteristics

Cells grew as monolayer cultures with a doubling time of about 23 h during logarithmic growth phase. When seeded into nonadhesive agar-coated petri dishes cells aggregated to each other. After 2–3 days cell aggregates were transferred into spinner culture flasks and cultivated as multicell spheroids. At about 390 μm multicell spheroids started to exhibit a necrotic inner zone which expanded with increasing diameter of the multicell spheroids whereas the thickness of the viable rim remained constant. All following experiments, therefore, were performed with multicell spheroids of diameters < 400 μm.

Stepwise trypsinization of multicell spheroids resulted in a concentric dissociation of the aggregates into three or four fractions, depending on the mean multicell spheroid diameter. Regardless of their origin in the multicell spheroid, cells of different fractions proliferated as monolayer cultures with the same lag phase and had similar doubling times (~23 h) as Ltk⁻ cells which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had only been cultivated as monolayers (Fig. 2). Interestingly, in serum-free medium cells proliferated faster under the three-dimensional growth condition and the extracellular volume increased from about 50% to about 65% of the total multicell spheroid volume.

β-Galactosidase Activity in Monolayer Cells

For more than 9 months flow cytometric measurements of monolayer cells revealed a constant β-galactosidase activity per cell as well as a stable portion of producing cells. These values did not change with increasing cell density in exhausted and in regularly renewed medium (Fig. 3). The portion of cells in G_0/G_1-phase, however, increased to ~60% when cells were cultivated without renewal of medium (Fig. 3a) whereas at the same cell density the portion of cells in G_0/G_1-phase was just 45% when medium was changed every 12 h (Fig. 3b). Our measurements indicate a constant β-galactosidase activity in monolayer cells without any influence by medium condition, cell density, or cell cycle phase.

β-Galactosidase Activity in Multicell Spheroids

In multicell spheroids the portion of producing cells decreased within 14 days from 98 to 2% as was measured by flow cytometry. The β-galactosidase activity in the remaining producing cells was unaffected (Fig. 4). Since the half-life of β-galactosidase (our unpublished results) is several days, FDG and X-gal have stained both cells which are still producing and cells which have already stopped producing β-galactosidase. The decrease of lacZ gene expression in these cells is, therefore, faster than indicated by Fig. 4 or Fig. 5. The loss of β-galactosidase-positive cells depended rather on the age than on the size of multicell spheroids: histological analysis of X-gal-stained cells showed in multicell spheroids of the same size a decrease of β-galactosidase-positive cells after different cultivation periods (Fig. 5). In multicell spheroids of different sizes but of the same cultivation period a constant portion of β-galactosidase positive cells was observed.

Transferring nonproducing multicell spheroid cells in monolayer culture resulted in a complete recovery of the β-galactosidase activity within a few days (Fig. 6) indicating that the decrease of β-galactosidase producing cells was not caused by a loss of the transfected lacZ gene. With clone M, a less pronounced age-dependent decrease of producing cells was observed (40% producing cells after 16 days) than that for clone A. The recovery of the β-galactosidase activity in monolayer culture was again similar as for clone A. With the other three clones, only histological investigations have been performed showing a similar decrease of β-galactosidase-positive cells as is demonstrated in Fig. 5.

The recovery could also be demonstrated in X-gal-stained 15-days-old multicell spheroids cultivated for 3 days in petri dishes where cells radially migrated from multicell spheroids. In cells located at a distance of ~200 μm from the multicell spheroids, β-galactosidase activity reappears and remains stable (Fig. 7). This transient decrease of β-galactosidase activity cannot be caused by a loss of the transfected lacZ gene; we, therefore, investigated the role of the transfected β-actin promoter.

β-Galactosidase Activity and Actin Concentration in Monolayer and Multicell Spheroid Cells

Since the expression of the transfected lacZ gene is regulated by its transfected β-actin promoter, both—the endogenous as well as the transfected—promoters could be influenced by the same cellular factors, leading to a reduced β-actin concentration and to a corresponding decrease in β-galactosidase activity. We, therefore, measured the β-galactosidase activity and the actin concentration simultaneously in monolayer cultures with different cell densities and in multicell spheroids. Figure 8 shows FITC–phalloidin-labeled monolayer cells where the filamentous actin is clearly stained. After a 20-fold increase in cell density (1.5 to 32 × 10^4 cells/cm^2) cells grown as monolayer exhibited only 60% of the original concentration of filamentous actin. Independent of cell densities the cellular β-galactosidase activity of FDG-stained monolayer cells remained constant (see Fig. 3). In cells of multicell spheroids the smallest concentration of filamentous actin was measured (46%). As already stated (Fig. 4), the β-galactosidase activity depended on the cultivation period. The noncorresponding changes of the β-galactosidase and the β-actin concentrations at different cell densities and cultivation periods may be due to different turnover rates for β-actin and β-galactosidase. Moreover, only the filamentous actin is labeled by the FITC–phalloidin method, whereas the globular actin is neglected. Our preliminary results from measurements of the total cellular actin concentration (Western blotting) did not indicate any differences in monolayers and multicell spheroids of different cultivation periods.

DISCUSSION

Tissue organization and functional cell–cell interactions have been studied in cell cultures by investigating multicell spheroids which reveal physiological and mor-

FIG. 5. Median sections of Ltk⁻ multicell spheroids after different cultivation periods. Stained with X-gal, hematoxylin/erythrosine. Dark staining indicates producing cells. (a) Three-day cultivation period; (b) 7 days; (c) 10 days; (d) 16 days; (e) 20 days; (f) 27 days. Bar: 50 μm.

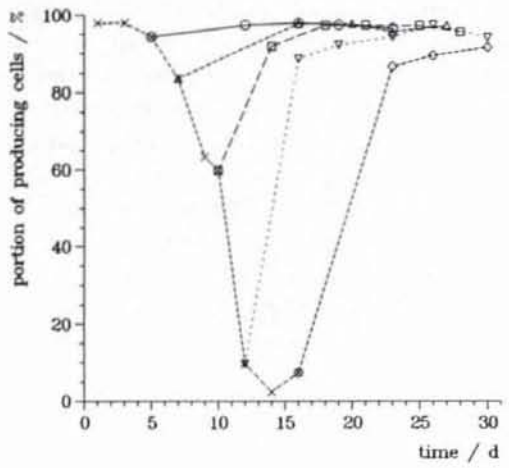

FIG. 6. Portion of β-galactosidase-producing Ltk⁻ cells in spheroids (×) and after retransferring the cells in monolayer culture (○, △, □, ▽, ◇).

phological qualities corresponding to *in vivo* tissues far better than the same cells cultivated as monolayers. For embryonic cells in three-dimensional cultures structural elements were well reestablished, indicating functional differentiation [1]. Tumor cells grown as multicell spheroids were found to increase their protein synthesis [24, 25], to be more resistant against radiation [20] and drugs [22], and to modify their gap junctional conductance according to the spheroid size [23, 32]. We have now found that the β-galactosidase production of transfected Ltk⁻ cells is downregulated in multicell spheroids whereas its production remained constant in monolayer cultures.

For all of these effects a multicomponent process seems to be more than likely. We have demonstrated that even in transfected cells where a constitutive promoter should ensure a constant gene product, such as β-galactosidase in our case, a permanent gene expression can not be taken granted. With the procedure used for transfecting Ltk⁻ cells, the lacZ gene is randomly integrated into the host genomic DNA. One might speculate that under these conditions the β-actin promoter is controlled by an additional regulatory element. For different clonal lines derived from a transfected population, different regulatory elements should then be operating. We have investigated, however, five different clones which responded qualitatively similar to the de-

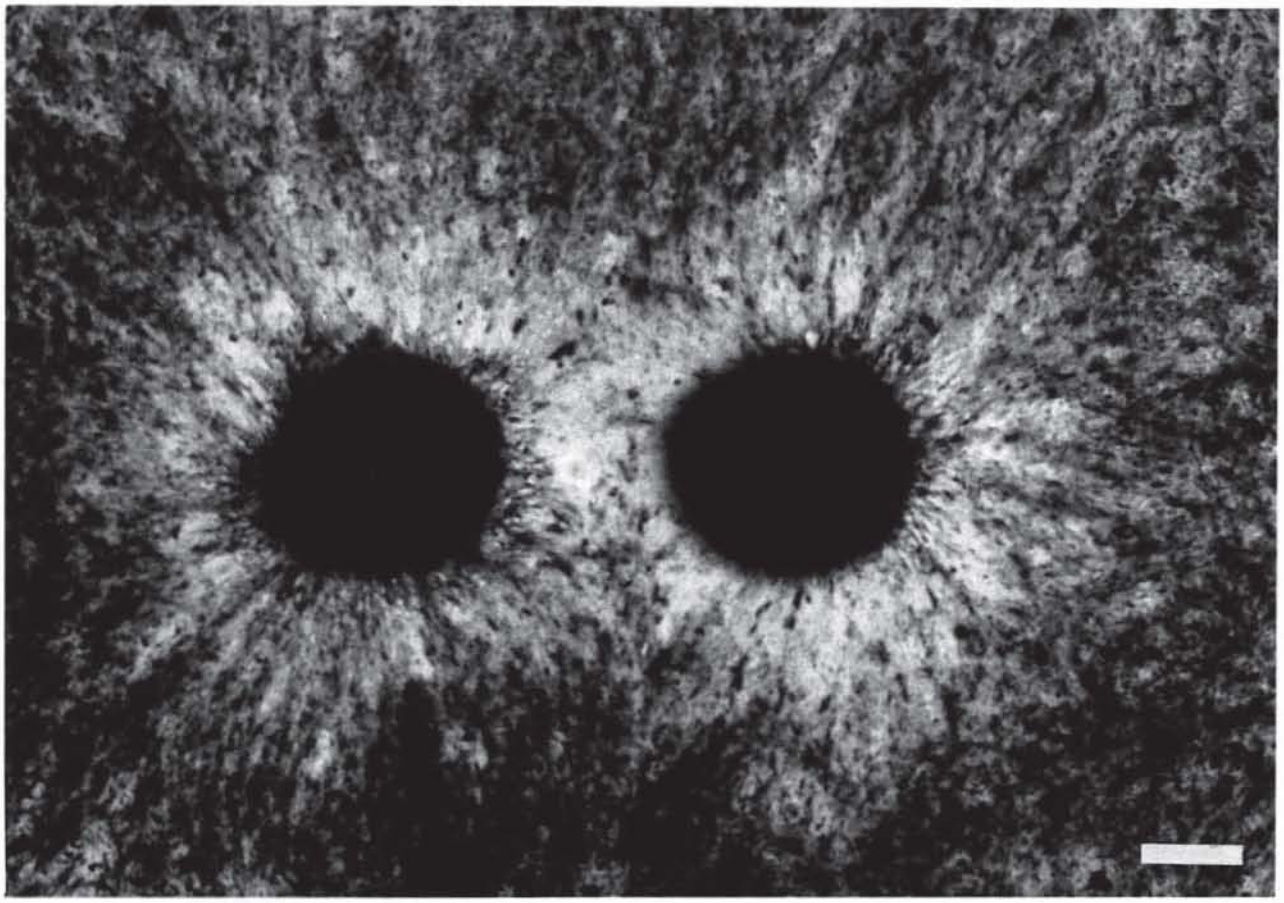

FIG. 7. Fifteen-day-old multicell spheroids cultivated in a petri dish for 3 days. Stained with X-gal, hematoxyline/erythrosine. Ltk⁻ cells radially migrated from the multicell spheroid. In monolayer cells dark staining indicates β-galactosidase activity. The darkness in the centers is due to the high density of the spheroids' cell mass. Bar: 50 μm.

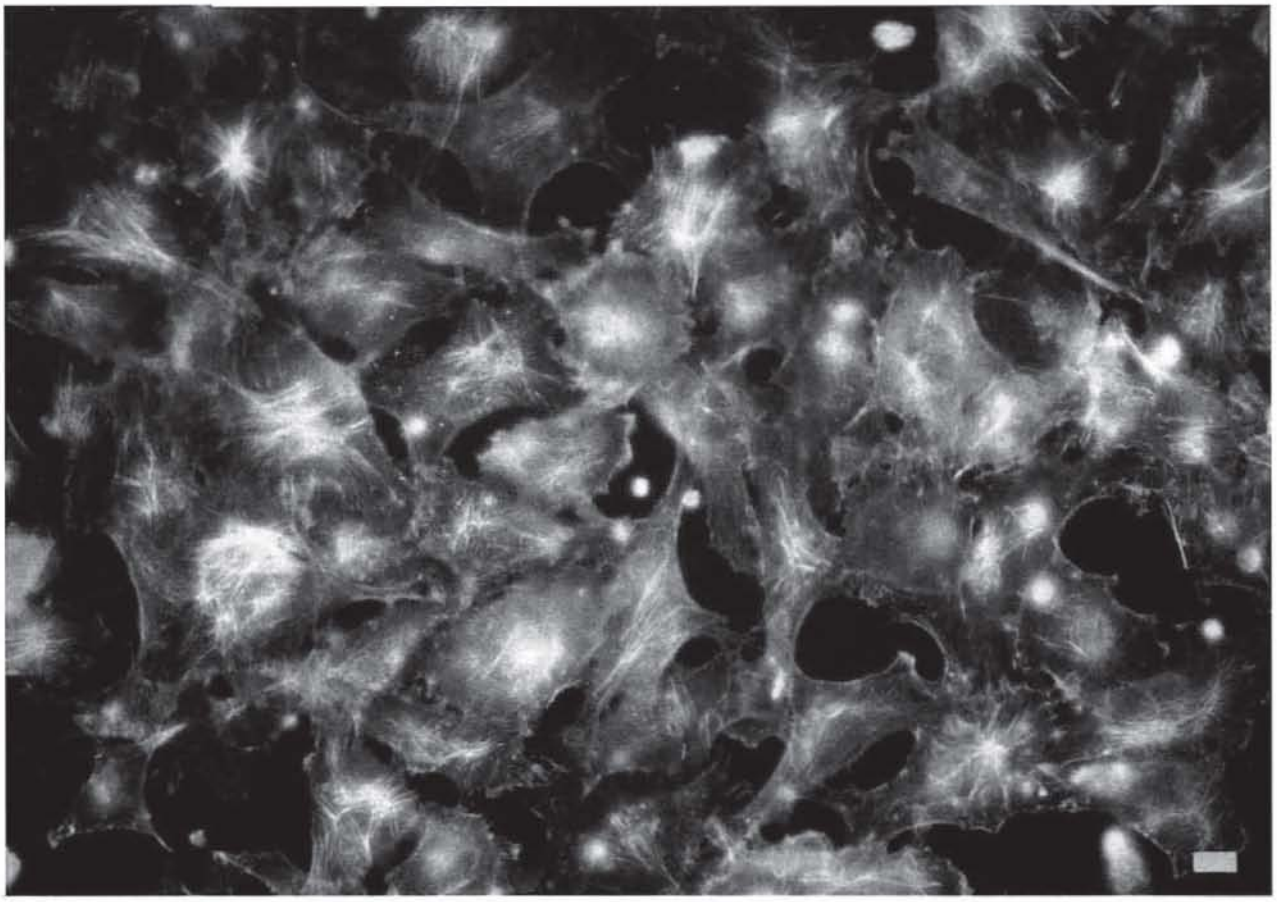

FIG. 8. Ltk⁻ cells grown as monolayer culture. FITC–phalloidin-labeled cells exhibit filamentous actin under epifluorescence illumination. Bar: 25 μm.

scribed clone A. This suggests an identical form of regulation which has been introduced with the lacZ gene. An unknown step in signaling path may have turned off the transfected human β-actin promoter whereas the endogenous β-actin expression seems not to be influenced. Cells in a multicell spheroid are characterized by a spherical shape whereas monolayer cells appear flattened with many filopodia. This different shape indicates also changes in the cytoskeleton of cells. Spherical cells contain no or considerably less actin fibers than flattened cells where bundels of actin filaments extend into filopodia and thus enable cellular locomotion [33] and adhesion to artificial substrates.

We are unable, however, to undoubtedly relate the observed cytoskeletal changes with the distinct reduction in β-galactosidase-producing cells. Not only do the turnover rates for the two proteins differ considerably (actin: ~8 h [34, 35]; β-galactosidase: several days [our unpublished data]) but also a reduced concentration of filamentous actin in cells grown in multicell spheroids does not allow the conclusion that the total amount of actin is reduced correspondingly. Our preliminary immunoblotting experiments revealed no differences in the total amount of actin between the two culture conditions. Under the experimental requirements for immunoblotting, the filamentous actin is disintegrated so that only globular actin is measured. Since actin is the most dominant protein in cells, minor changes will not be resolved. Furthermore, we cannot exclude that the transfected human promoter follows different regulatory pathways than the endogenous mouse promoter. Under these conditions, therefore, a correlation between β-galactosidase activity and actin concentration could not be observed. In any case, we have added new evidence for another regulatory event which is only triggered under the specific conditions that are established by the *in vivo*-like three-dimensional organization of permanently growing cells.

We thank Ms. Beate Rehkopf for expert technical assistance, Ms. Katrin Klein for evaluating the cell cycle phases, Dr. Hansjörg Hauser, GBF Braunschweig, for supporting us with the transfected Ltk⁻ cells, and Reiner Eckert for helpful discussions. I. K. gratefully acknowledges a scholarship from the Graduiertenförderung des Landes Baden-Württemberg.

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Received November 23, 1992
Revised version received March 16, 1993