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Establishment of Two Rabbit Mammary Epithelial Cell Lines with Distinct Oncogenic Potential and Differentiated Phenotype after Microinjection of Transforming Genes

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The goal of this work was to establish an assay for transformation of epithelial cells. Two epithelial cell lines were obtained after microinjecting transforming genes into primary rabbit mammary secretory cells. The cell lines were analyzed for their oncogenic potential and for the maintenance of a differentiated phenotype. A fully transformed cell line, which retained epithelial cell organization, was obtained by coinjecting simian virus 40 DNA and the activated human c-Ha-*ras* gene. The proliferation rate of these cells was high, with a doubling time of 16 h. Their growth was anchorage independent, and they had lost contact inhibition. The cells were tumorigenic in nude mice, but had no metastatic potential. Both microinjected DNAs were efficiently transcribed and translated, in contrast to the casein genes, which were expressed in primary cells but not in the transformed cell line. An immortalized cell line established after injection with simian virus 40 DNA alone was characterized by a moderate rate of proliferation with a doubling time of ~30 h. The growth of these cells was contact inhibited and anchorage dependent. The cells were not tumorigenic in nude mice.The viral DNA was expressed during early passages, as shown by the presence of the large T antigen in cell nuclei, but not at later passages. A high number of lactogenic hormone receptors were found associated with the cell surface. Despite the presence of these receptors, no induction of genes coding for milk proteins was observed after addition of prolactin. These data demonstrate that this assay system can be used to assess the immortalizing and transforming potential of candidate oncogenes in epithelial cells.

Transformation of fibroblast cell lines by oncogenes or DNA from tumors results in morphological alteration, changes in growth properties, and sometimes tumorigenicity (14, 15, 30). In this situation a single oncogene can be sufficient to induce transformation, whereas the action of at least two oncogenes was shown to be required for primary rat embryo fibroblasts or embryonic cells to acquire a transformed phenotype and become tumorigenic (15, 23). Such observations led to the concept that transformation of primary fibroblasts proceeds in multiple steps resulting in an increased life span of cells, immortalization, the appearance of a transformed phenotype in culture, and tumorigenicity. Whether primary epithelial cells undergo similar stepwise changes under the control of oncogenes remains to be established. Several spontaneously growing epithelial cell lines have been established from normal murine mammary tissue (5, 21, 32). Such established mammary epithelial cell lines have been successfully transformed by infection with simian virus 40 (SV40) (2) and by transfection with genomic DNA from the human EJ bladder carcinoma cell line which contains an activated c-Ha-*ras* oncogene (12). Transformation of primary mammary epithelial cells has been observed after infection of human (4) and murine (2) cells with SV40.

To assess the potential of known and candidate oncogenes to transform mammary epithelial cells, we developed an assay system with primary rabbit mammary epithelial cultures as target cells. Mammary tumors are very rare in rabbits, and rabbit mammary cells appear to be unable to undergo spontaneous transformation. In the mouse, analysis of the oncogenic potential of various oncogenes in mammary epithelial cells is complicated by the presence of endogenous mouse mammary tumor virus sequences, which may be activated by the transfection procedure and participate in the immortalization or transformation process. In the rabbit no mammary tumor virus-like elements have been described.

We chose primary mammary cells from rabbits in midpregnancy as target cells and studied the action of various oncogenes on their growth properties and the expression of their tissue-specific functions. Primary cultures were grown on different substrates in a chemically defined medium under conditions in which the growth properties and pattern of expression of milk protein genes in response to hormonal stimulation were shown to be comparable to those in vivo (10, 28). To test the sensitivity of the cells to transformation, we used papova virus (SV40) DNA and activated human c-Ha-*ras* DNA. In this paper, we describe two cell lines established by microinjecting SV40 DNA alone and in combination with the activated human c-Ha-*ras* oncogene. An immortalized cell line obtained after injecting SV40 DNA into primary cells retained some but not all of the differentiation markers of mammary secretory cells from pregnant rabbits, whereas a cell line fully transformed by SV40 and the activated human c-Ha-*ras* DNA became tumorigenic.

MATERIALS AND METHODS

Primary cultures of rabbit mammary epithelial cells. Mammary epithelial cells obtained from rabbits in

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midpregnancy (13) were cultured by published procedures (10). Briefly, cells pooled from different rabbits were plated on Primaria plastic dishes (Falcon Plastics) at a density of 1 $\times 10^4$ to 2 $\times 10^4$ cells/cm^2 and cultured in M199-F12 medium (1:1) containing 10% horse serum and 5% fetal calf serum (FCS). After 24 h, the medium was replaced by a serum-free medium complemented with dexamethasone (1 nM), 17 β-estradiol (100 pM), triiodothyronine (1 nM), epidermal growth factor (10 ng/ml), prostaglandin F2α (10 ng/ml), insulin (5 μg/ml), and 0.25% bovine serum albumin. When required, bovine prolactin (a gift from C. M. Li, San Francisco) (5 nM) was added to the medium. The primary cells were cultivated for 8 to 10 days in serum-free medium before microinjection. Eight days after microinjection the cells were trypsinized, plated onto 3.5- or 6-cm plastic dishes, and cultured for 12 h in serum-containing medium (5% FCS) and then in serum-free medium. The cells were trypsinized each week or every other week, depending on the proliferation rate, and plated at a 1:2 dilution until the passage 5, 1:3 until passage 15, and then at a 1:3 to 1:5 dilution. Control primary mammary cells did not survive after two to three passages. Trypsinization was started by adding 0.05% trypsin–20 mM EDTA for about 10 min and stopped by the addition of F12-M199 medium containing 5% FCS. The cells were dispersed by pipetting, diluted, plated, and cultivated for 4 to 12 h in serum-containing medium. Cultures on collagen gels were done as described previously (10).

Microinjection of DNA. SV40 DNA (provided by P. Beard) was precipitated in ethanol and suspended in 10 mM Tris hydrochloride, pH 7.1, at 0.25 mg/ml. Plasmid pEJ, containing the human tumor c-Ha-*ras* gene (25), was suspended in 10 mM Tris hydrochloride, pH 7.1, at 0.5 mg/ml.

Microcapillary injection of cloned DNAs was performed under a Zeiss phase contrast microscope (200× magnification) equipped with a Leitz micromanipulator (8) and the automatic injector developed in our laboratory (Inject-Matic; Gabay Electronique, Geneva). About 1 pl of DNA solution was injected into each nucleus.

Anchorage dependence assay. Cells were plated between two layers of agarose (0.5% agarose in the bottom layer and 0.3% in the upper layer) at 10^3, 10^4, 5×10^4, and 10^5 cells per 10-cm plate and cultured in the presence of 5% FCS or in serum-free medium. After 10 days, the colonies which formed were picked and grown in serum-free medium.

Immunofluorescence. Cells grown on plastic were fixed with acetone-methanol (1:1), washed with phosphate-buffered saline, and incubated with either hamster anti-SV40 T antigen (a gift from H. Türler, University of Geneva) or with antikeratin monoclonal antibody L61 (a gift from B. Lane, London). After being washed, the cells were incubated with fluorescein-conjugated rabbit anti-hamster immunoglobulin G (IgG) or biotinyl horse anti-mouse IgG and fluorescein isothiocyanate-conjugated streptavidin, respectively. Cell preparations were observed with a Zeiss photomicroscope with UV epifluorescence.

Nucleic acid extraction. Cultured cells were recovered from plastic dishes by trypsin-EDTA treatment and from collagen gels by collagenase digestion. Total cellular RNA was extracted, polyadenylated and [poly(A)$^+$] RNA was isolated by oligo(dT)-cellulose chromatography (17). Genomic DNA was recovered from a cell pellet by treatment with 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg of proteinase K per ml by phenol-chloroform (1:1) extraction and ethanol precipitation. Contaminating RNA was removed by RNase A (20 μg/ml) digestion for 1 h at 37°C. This treatment was followed by further digestion with proteinase K and two phenol-chloroform extractions. The DNA was finally dissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA and stored at 4°C.

Filter hybridization. DNA samples (10 μg) were digested with different restriction enzymes for 14 h at 37°C, electrophoresed in 0.85% agarose gels, and transferred onto nitrocellulose filters (26). Filters were hybridized for 24 h at 65°C with ^{32}P-labeled, nick-translated probes in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM sodium phosphate buffer (pH 6.5)–1× Denhardt solution–0.05% SDS–0.005% sodium pyrophosphate–125 μg of calf thymus DNA per ml. Filters were washed for 2 h at 65°C in 0.1× SSC–0.05% SDS–0.01% sodium pyrophosphate, dried, and exposed to XAR-5 film at −70°C for various times.

Poly(A)$^+$ RNA was glyoxalated, electrophoresed in 1.1% agarose gels, and transferred to nitrocellulose filters by the method of Thomas (31). Hybridization with ^{32}P-nick-translated probes was carried out at 42°C for 48 h in 50% formamide–5× SSC–50 mM sodium phosphate buffer (pH 6.5)–1× Denhardt solution–0.2% SDS–10 mM EDTA–50 μg of calf thymus DNA per ml. Filters were washed for 2 h at 50°C in 0.1× SSC–0.1% SDS, dried, and exposed to XAR-5 film at −70°C for various times.

Hormone-binding assays. Lactogenic hormone-binding sites on dispersed cells were determined as described previously (27) with human growth hormone (HGH) as the ligand. The assay was carried out at 4°C to prevent hormone internalization. Scatchard plot analysis was performed with the Scatfit program developed by Munson and Rodbard (18).

RESULTS

Microinjection of oncogenes into primary mammary epithelial cells. Our attempts to transform or immortalize rabbit mammary epithelial cells by transfecting various oncogenes by calcium phosphate precipitation (9) or protoplast fusion (24) failed, mainly because fibroblasts that were more resistant to the toxic effects of these procedures rapidly overgrew the epithelial cell population. Therefore, selective microinjection of DNA into the nuclei of subconfluent epithelial cells was chosen to overcome these problems. We introduced either SV40 DNA, the EJ plasmid containing the c-Ha-*ras* gene isolated from human bladder carcinoma cells (25), or both. Approximately 100 circular molecules of SV40 DNA or pEJ plasmids per cell were introduced into primary epithelial cells. In cultures injected with both DNAs, cell proliferation was observed in two different areas of the culture dish (minimum of 2 clones per 200 cells). One of these clones was established as a cell line. The cells exhibited new growth properties, with a doubling time of 16 h, compared with 72 h for primary cells under identical conditions (28). These cells, referred to as transformed mammary cells, have been maintained in continuous culture for more than 1 year and have been passaged over 65 times.

Cells injected with SV40 DNA alone (2 clones per 500 cells) also changed their growth properties, but their proliferation rate was slower, with a doubling time of ~30 h. These cells, referred to as immortalized mammary cells, have also been maintained in culture for more than 1 year and have been passaged ~40 times. Finally, cells microinjected with c-Ha-*ras* DNA alone divided once or twice after the manipulation but neither formed clones nor survived in culture. No spontaneously proliferating cell clones were observed in mock-injected cultures.

Morphology of the two mammary cell lines. The transformed mammary cells were cloned by limiting dilution

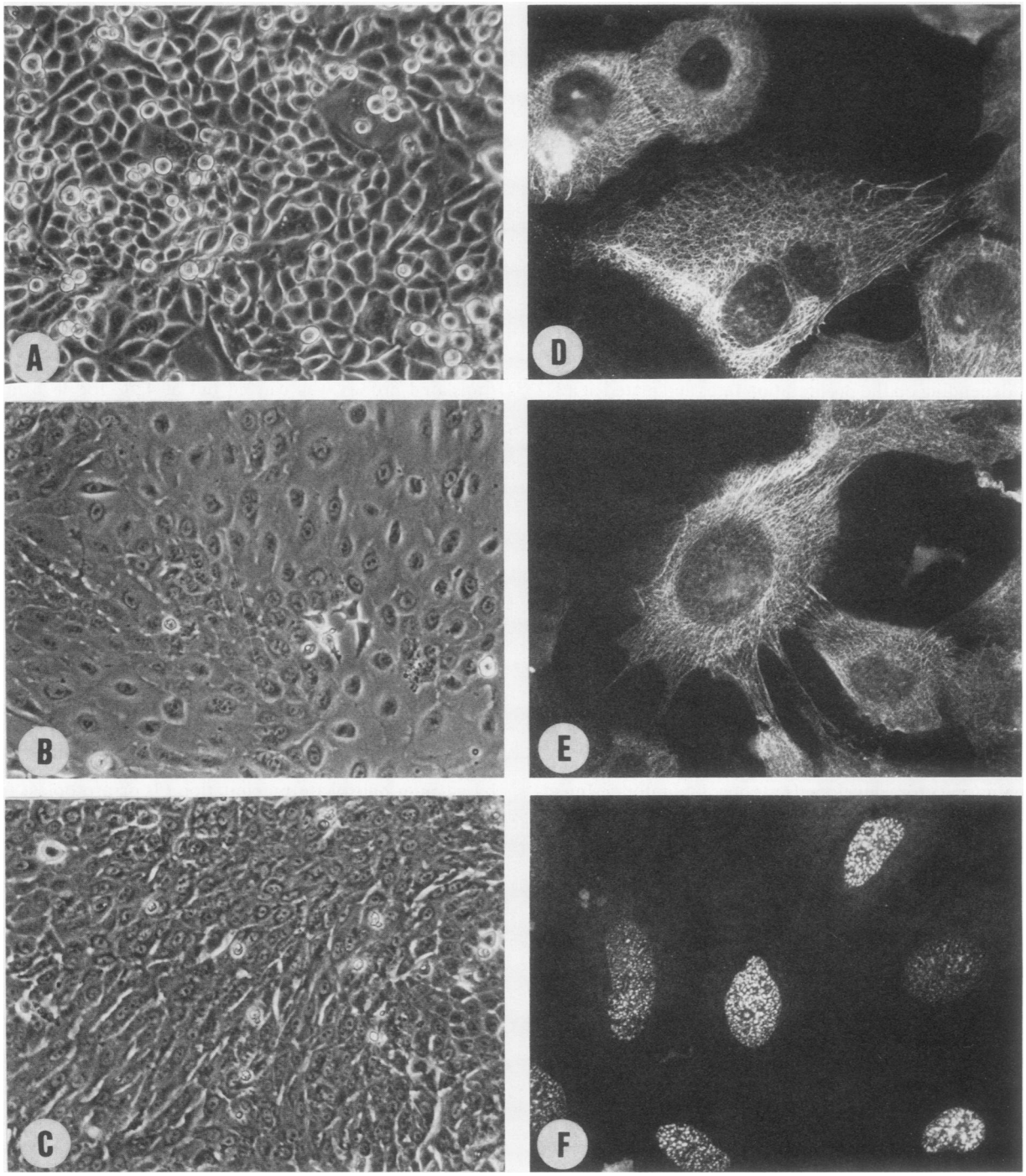

FIG. 1. Phase-contrast light (A, B, and C, ×150) and immunofluorescence (D, E, and F, ×400) micrographs of immortalized and transformed rabbit mammary epithelial cells. (A) Transformed cells at passage 5, cuboidal shape. (B) Immortalized cells at passage 4. (C) Primary mammary cells after 3 weeks in culture. (D and E) Detection of keratin filaments with monoclonal antibody L61 specific for cytokeratin 18 (45 kilodaltons [16]) in transformed (D) and immortalized (E) cells. (F) Detection of the SV40 large T antigen in transformed cells.

(cloning efficiency, ~30%). The cloned cells were grown on plastic or embedded in collagen gels for analysis of their functional properties and in soft agar to test their anchorage dependency. On plastic, two distinct morphological phenotypes were observed. Most cells were cuboidal (Fig. 1A) and a few were elongated. During subculture, conversion of cuboidal cells into elongated cells was observed, and this morphological change was stable after further culture. The cells grown to confluency on plastic were unable to form domes, a structure characteristic of differentiated mammary cells in culture, reflecting vectorial transport of solutes and water. The cells rapidly became multilayered, as shown by electron microscopy (Fig. 2A). The superficial cells in contact with the medium retained cell surface polarity with

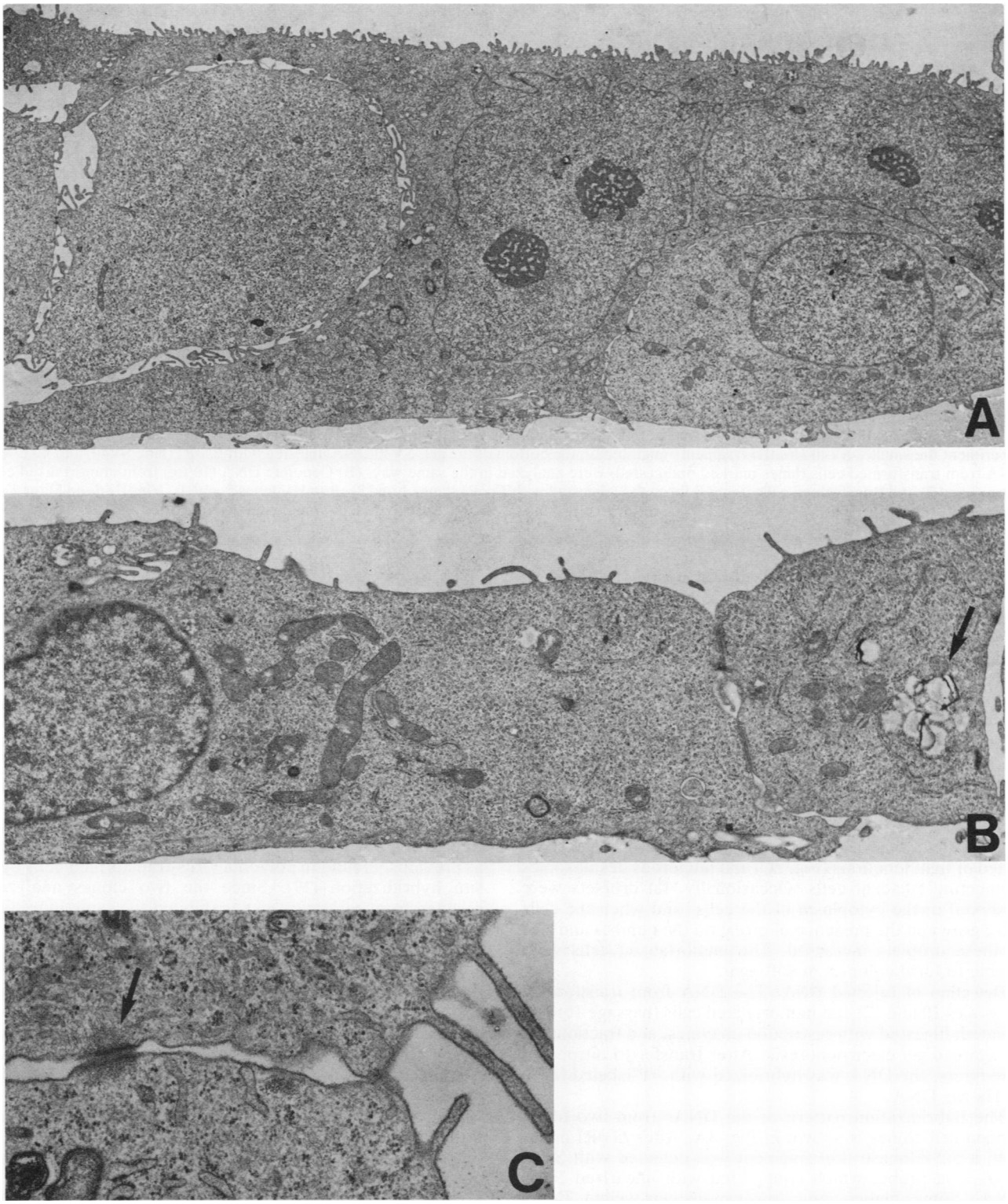

FIG. 2. Electron micrographs of transformed (A) and immortalized (B and C) mammary cells. The cells were grown on attached collagen gels for 10 days in the presence of 2% FCS. Sections perpendicular to the plane of the monolayer are shown. Microvilli were identified in both cell lines. (A) Transformed cells formed multilayers. (B) Accumulation of fat droplets in immortalized cells (arrow). (C) Junctional complexes, including desmosomes (arrow) and tight junction (inset) in immortalized cells.

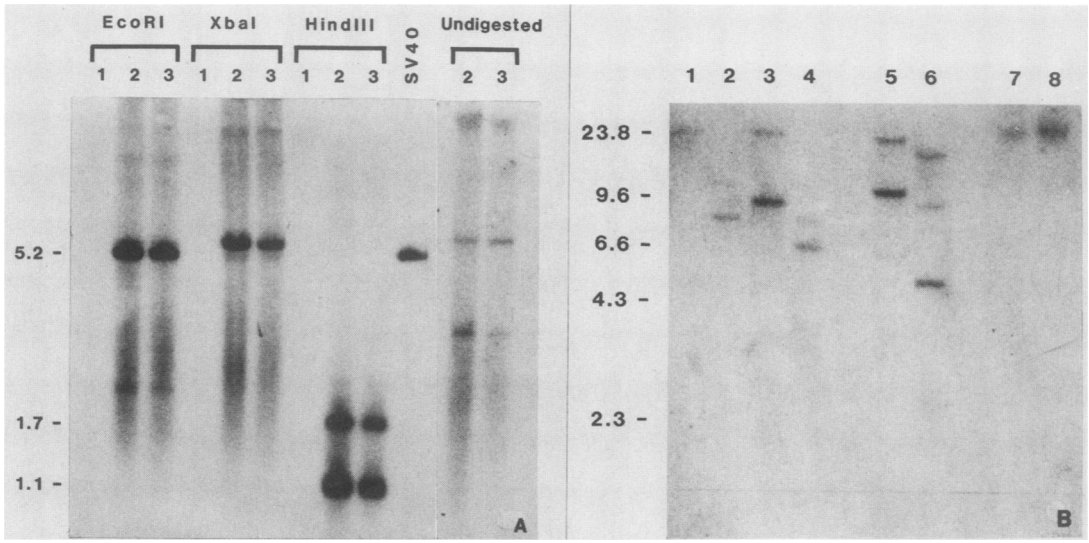

FIG. 3. Analysis of SV40- (A) and Ha-*ras*- (B) specific DNA sequence content in the genome of microinjected cells. (A) Genomic DNA (10 μg) from the immortalized mammary cell line (lanes 1) and from two transformed cell lines (lanes 2 and 3) digested with *Eco*RI, *Xba*I, or *Hin*dIII were electrophoresed in a 0.3% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled SV40 DNA. In this experiment the smaller SV40 *Hin*dIII fragments migrated off the bottom of the gel. SV40 DNA digested with *Eco*RI (lane SV40) and high-MW DNA from transformed cells (lanes marked undigested) were analyzed in the same way. (B) Genomic DNA (10 μg) from immortalized cells (lanes 1 and 2) and transformed cells (lanes 3 through 8) digested with *Eco*RI (lanes 1 and 3), *Bam*HI (lanes 2 and 4), *Hin*dIII (lane 5), or *Kpn*I (lane 6) or undigested high-MW DNA from transformed cells (lanes 7 and 8). In both panels, fragment sizes (in kilobases) are shown to the left.

numerous microvilli associated with the apical plasma membrane (Fig. 2). Desmosomes, but no tight junctions, bridged adjacent cells. The epithelial characteristics of the cells were further substantiated by analyzing the type of intermediate filaments which were expressed by using monoclonal antibody L61, directed against keratin (16). The labeling pattern was typical of that described for epithelial cells (4). When grown in soft agar, the transformed mammary cells formed colonies with high efficiency (~10%).

The immortalized mammary cells were of epithelial morphology (Fig. 1B), contained cytokeratin intermediate filaments (Fig. 1E), and formed monolayers at confluency. They were polarized, with long microvilli present on the apical plasma membrane (Fig. 2B). The junctional complex consisted of tight junctions (Fig. 2C) and numerous desmosomes connecting adjacent cells. Occasionally, fat droplets were observed in the cytoplasm of the cells, and when the cells were grown in the presence of prolactin the number and size of these droplets increased. The immortalized cells were unable to form colonies in soft agar.

Detection of injected DNA. The DNA from transformed (passages 10 and 12) and immortalized cells (passage 10) was isolated, digested with restriction enzymes, and fractionated by agarose gel electrophoresis. After transfer to nitrocellulose filters, the DNA was hybridized with ^{32}P-labeled SV40 or Ha-*ras* DNA.

The hybridization pattern of the DNAs from two transformed cell clones is shown in Fig. 3A. After *Eco*RI digestion, a 5.2-kilobase (kb) fragment was detected with SV40 DNA as a probe, which comigrated with linearized SV40 DNA. Some higher- and lower-molecular-weight (MW) bands were also labeled, although less intensely. These bands probably represented SV40 DNA integrated into genomic DNA. Digestion with *Bam*HI or *Bam*HI and *Xba*I generated a similar pattern (data not shown). In contrast, the enzyme *Xba*I, which does not cleave SV40 DNA, gave rise to a distinct band which did not comigrate with linear SV40 DNA, but behaved like circular, unintegrated SV40 DNA. This was confirmed by extraction of the low-MW DNA (11) and hybridization with the same probe. The same band, migrating more slowly than linearized SV40 DNA, was detected. Finally, digestion of the DNA from transformed cells with the enzyme *Hin*dIII generated the same fragments as those obtained from the digestion of SV40 DNA.

The number of copies was estimated by comparing the intensity of hybridizing bands with standards containing a known amount of SV40 DNA. Thus, we estimated that transformed rabbit cells contained three to four integrated DNA molecules and 10 to 14 copies of free SV40 DNA per cell. Rabbit cells are semipermissive for SV40 (1, 33), and free viral DNA may accumulate in a minor fraction of cells as previously found in polyoma virus-transformed cells by in situ hybridization (19). Since the two clones analyzed, selected from early passages, exhibited the same hybridization pattern, they were probably derived from a single transformed cell.

In the immortalized cells injected with SV40 DNA alone, the large T antigen was detected in 5 to 8% of the cells during early passages (data not shown), indicating the presence and expression of the microinjected DNA. After eight passages the large T antigen was no longer observed, and at the 10th passage no viral DNA sequences were detected (Fig. 3A, lane 1).

To detect the presence of microinjected c-Ha-*ras* in transformed mammary cells, cellular DNA was hybridized with the BS-9 (460 base pairs) fragment of Harvey murine sarcoma virus DNA (6). A 24-kb *Eco*RI and an 8.5-kb *Bam*HI fragment containing the endogenous rabbit cellular *ras* gene were identified in both normal rabbit mammary cells and immortalized cells (Fig. 3B, lanes 1 and 2). In transformed cells, however, there were additional bands, a 9.5-kb *Eco*RI and a 6.6-kb *Bam*HI fragment (lanes 3 and 4). A similar pattern was obtained after *Hin*dIII digestion (Fig. 3B, lane 5), showing two bands containing the endogenous rabbit *ras*

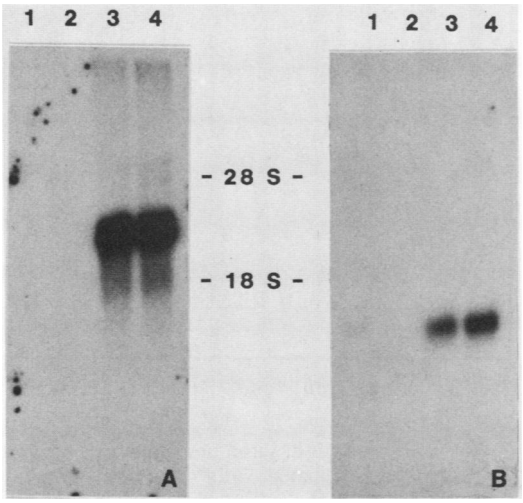

FIG. 4. Analysis of SV40- (A) and Ha-*ras*- (B) specific RNAs expressed in transformed mammary cells. (A) Poly(A)$^+$ RNA (7 μg) from rabbit liver (lane 1), rabbit mammary glands (lane 2), and transformed cells cultured on collagen gels in the absence or presence of prolactin (lanes 3 and 4) were electrophoresed, transferred to nitrocellulose filter, and hybridized with ^{32}P-nick-translated SV40 DNA. (B) The filter shown in A was hybridized with the ^{32}P-labeled BS-9 460-base-pair fragment of the Ha-*ras* plasmid. RNA sizes are indicated.

sequences and an additional 10.9-kb band corresponding to the injected human c-Ha-*ras* EJ plasmid. Two additional bands (9.3 and 5.2 kb) were detected in DNA digested with *Kpn*I (Fig. 3B, lane 6), an enzyme which cleaves the human c-Ha-*ras* coding sequence into two fragments. When undigested DNA was analyzed, the c-Ha-*ras* sequences migrated with the high-MW DNA (lanes 7 and 8). By comparison of band intensities with standards, we concluded that three to four copies of the EJ plasmid containing the activated human c-Ha-*ras* gene were present in the rabbit cell nuclei in the form of tandem repeats.

Expression of microinjected genes in transformed mammary cells. To test whether the microinjected DNAs were expressed in the transformed mammary cells, total RNA was extracted from cells grown on floating collagen in the presence or absence of prolactin. The poly(A)$^+$ RNA was recovered by oligo(dT)-cellulose column chromatography, size fractionated by agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled SV40 DNA or with the BS-9 Ha-*ras* fragment. High levels of SV40 mRNA accumulated in the transformed cells in the presence and the absence of prolactin (Fig. 4A, lanes 3 and 4). In rabbit liver and mammary gland control RNAs, no SV40-specific sequences were detected (Fig. 4A, lanes 1 and 2). When the poly(A)$^+$ RNA was probed with the nick-translated Ha-*ras* sequence (Fig. 4B), a low level of *ras*-related RNA (~1.2 kb) was detected in both liver (lane 1) and mammary gland (lane 2) cells. However, in transformed cells the amount of *ras*-specific RNA was much higher (lanes 3 and 4). The presence of prolactin in the culture medium slightly increased the level of *ras* RNA accumulation (Fig. 4B, lane 4). The SV40 mRNA was translated, as shown by the presence of T antigen in the cell nuclei (Fig. 1). In conclusion, both microinjected oncogenes were expressed in transformed cells, whereas in immortalized cells no expression of SV40 genes could be detected after the eighth passage.

Tumorigenicity of the two mammary cell lines. The tumorigenic potential of the transformed and immortalized cells was analyzed by inoculating 5×10^6 cells into nude mice and scoring the development of subcutaneous tumors or metastases. As a control, nude mice were injected with the same number of primary mammary cells. Primary cells did not induce the formation of tumors even after a 3-month period (Table 1). In one of six nude mice inoculated with the immortalized cells, a progressive tumor appeared after 1 month. In contast, tumors developed after 6 to 8 days when transformed cells were injected. There was no difference in the tumorigenic potential between transformed cells originating from uncloned populations and populations cloned in soft agar or collagen gels. In tumor-bearing animals, no metastases were detected by histological examination of regional lymph nodes and lungs. The tumors, analyzed by light and electron microscopy, were composed of undifferentiated carcinoma cells (Fig. 5), with scattered areas reminiscent of glandular structures. The cells were heterogeneous in shape and remained polarized with the presence of desmosomes, but no tight junctions were found. An extracellular matrix material lined the space between the mouse stroma and the tumor cells. Tumors were passaged twice in nude mice and then dissociated mechanically, and the cells were cultured in vitro. Their morphology and growth properties remained identical to those of the transformed cells; they also expressed the SV40 large T antigen, confirming that they derived from the inoculated transformed rabbit cells.

Expression of mammary-specific genes. Primary mammary cells from rabbits in midpregnancy are able to proliferate and differentiate in vitro under appropriate culture conditions (10, 28). For such cells to express milk protein genes, lactogenic hormone receptors must be present and the cells must be grown on flexible substrata (7). Since the cells which were transformed or immortalized were obtained from the glands of pregnant rabbits, we tested whether they expressed lactogenic hormone receptors and were able to express milk protein genes in response to prolactin stimulation. Transformed and immortalized cells were grown under various conditions and harvested at different passages for receptor determination by a radioreceptor assay (27). Transformed cells were able to specifically bind lactogenic hormones (ovine prolactin or HGH), but the level was too low to allow Scatchard plot analysis. In addition, the binding capacity progressively decreased with passage number. In immortalized cells the binding capacity was much higher, allowing Scatchard plot analysis (Fig. 6). When grown on plastic and in presence of 2% FCS, these cells expressed a single class of high-affinity (K_A, 8×10^9 M^{-1}) binding sites at

TABLE 1. Tumorigenicity of mammary cells in nude mice

Cells	Cells injected (10^6/mouse)	No. of mice with tumors/no. injected		
		Days 6–8	Day 30	Day 120
Primary mammary	5	0/2	0/2	0/2
Immortalized	5	0/6	1/6	1/6
Transformed	5	5/5	5/5	—[c]
Transformed clone A[a]	5	1/1	1/1	—
Transformed clone C[b]	5	1/1	1/1	—

[a] Transformed cell clone A was isolated from agarose.
[b] Transformed cell clone C was isolated from collagen gel.
[c] —, No survivors.

the level of ~1,600 receptors per cell, a number twofold higher than the number expressed on cells from pregnant animals (27).

Transformed and immortalized cells were grown for 2 weeks on attached collagen gels in the absence of prolactin. The collagen gels were then detached and the cells were further cultivated on floating gels in the presence of 5 nM prolactin for another 2 weeks. The cells were treated with collagenase and lysed, and total mRNA was isolated, electrophoresed, transferred to nitrocellulose filters, and hybridized with a rabbit α-casein probe (29). No accumulation of casein mRNA was detectable (Fig. 7) in either transformed cells or immortalized cells. We also tested, by Western blotting and immunoprecipitation of [^{35}S]methionine-labeled cell extracts, whether α- and β-caseins and transferrin were synthesized in the transformed or immortalized cells. No milk proteins were recovered from the cells or from the medium, but transferrin was immunoprecipitated from labeled transformed cells. Thus, despite the presence of lactogenic hormone receptors, the immortalized mammary cells did not express prolactin-inducible milk protein genes.

DISCUSSION

Primary mammary cells isolated from pregnant rabbits served as target cells to study the oncogenic potential of a cellular oncogene, the activated human c-Ha-*ras* gene, and of SV40 DNA. When injected alone, these molecules were unable to transform rabbit mammary cells. The combination of SV40 DNA and the activated c-Ha-*ras* gene, however, induced drastic changes in the microinjected cells. The proliferation rate increased fourfold over that of primary cells cultivated under identical conditions (28). The cell growth became anchorage independent and the cells were tumorigenic in nude mice. Both genes were expressed in the transformed cells. A few SV40 DNA copies were integrated, whereas most molecules were present as unintegrated, probably autonomously replicating circles in the semipermissive rabbit cells (33). The SV40 DNA was transcribed and expressed as evidenced by the presence of T antigen in the majority of cell nuclei. The activated human c-Ha-*ras* gene was present in the form of four tandem repeats most likely integrated in the cellular genome.

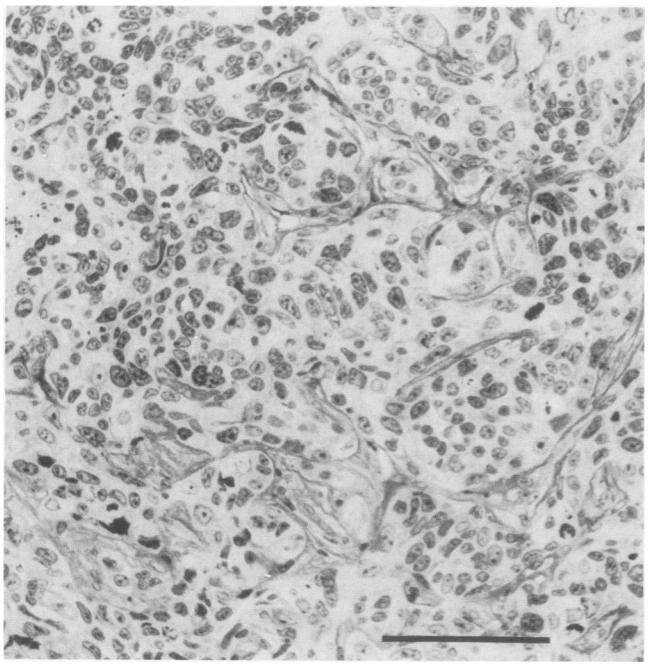

FIG. 5. Tumor obtained in nude mice following subcutaneous injection of transformed rabbit mammary cells. The undifferentiated tumor consisted of solid carcinoma cell cords lined by connective and vascular mouse stroma. Note numerous mitotic figures. Giemsa staining on plastic-embedded 2-μm-thick section. Bar, 100 μm.

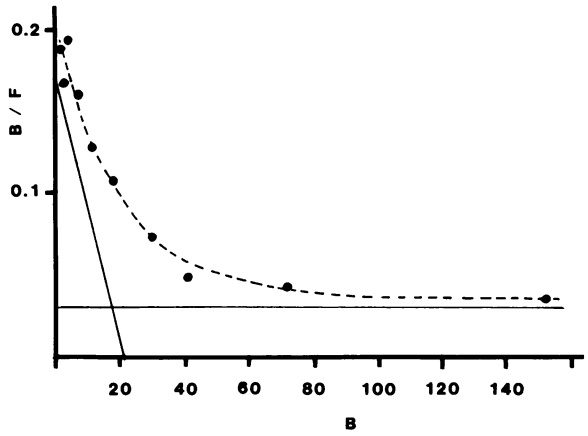

FIG. 6. Scatchard analysis of saturation binding curve for HGH. Cells (4×10^6) were incubated with ^{125}I-HGH and increasing amounts of unlabeled hormone. Abbreviations: B, bound hormone (in picomoles); B/F, bound hormone-free hormone ratio. Nonspecific binding was not substracted and appears as a small asymptote (nonsaturable sites) on the Scatchard graph. Points represent mean values of triplicate assays. The number of binding sites was 1,589, and the affinity constant K_A was 8×10^9 M^{-1}.

The probe which was used to analyze *ras* gene expression did not discriminate between the transcripts from the activated human and the endogenous rabbit genes. It cannot be formally excluded that transformation resulted from enhanced transcription of the rabbit c-*ras* gene, but it is more likely that the change of growth properties and the acquisition of tumorigenicity were dependent on expression of the mutated human *ras* gene, as has been shown in other systems (3, 12). Cooperation between the *ras* gene and SV40 was required for expression of the transformed phenotype, since each gene alone was ineffective. With the *ras* gene alone, no clones were observed, suggesting that the *ras* gene product alone is unable to prolong the life span of rabbit mammary cells. These results are compatible with the observations reported previously for transformation of primary rat embryo fibroblasts (15). It is possible that in the transformed mammary cells, the SV40 large T antigen or other SV40 gene products allowed the cells to proliferate and rendered them responsive to the action of the mutated c-Ha-*ras* gene product, which by itself was ineffective. In contrast, microinjection of SV40 DNA did change the proliferation rate of rabbit mammary cells. Although several attempts to maintain primary mammary epithelial cells in culture failed, after SV40 DNA microinjection a cell line was established and cultivated for more than a year. In this cell line, the large T antigen of SV40 was detected in early passages; however, after the 10th passage, neither viral DNA nor viral gene products were detectable, suggesting that the injected DNA had been eliminated and that maintenance of the immortalized phenotype was not dependent on the continuous presence and expression of SV40 DNA. It remains possible that a small piece of SV40 DNA, for

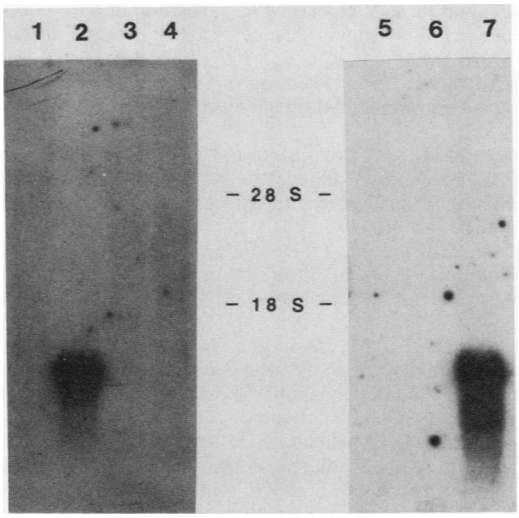

FIG. 7. Expression of casein genes in transformed and immortalized mammary epithelial cells. Transformed and immortalized mammary cells were grown attached on collagen gels for 2 weeks and detached for 2 additional weeks in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of 5 nM prolactin. Poly(A)$^+$ RNA (7 μg) from rabbit liver (lane 1), transformed cells (lanes 3 and 4), and immortalized cells (lanes 5 and 6) and total RNA (1 μg) from rabbit mammary glands (lanes 2 and 7) were electrophoresed in a 1.1% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled casein probe (29). RNA sizes are indicated.

example a transcriptional enhancer or a promoter, was integrated at a site in the host chromosome which activated genes involved in regulating cell proliferation. Even after more than 35 passages, the immortalized cells did not express a transformed phenotype; they remained anchorage dependent and did not cause early tumors in nude mice (Table 1).

Since the two established cell lines exhibited distinct growth properties and oncogenic potentials, we examined at which stage of differentiation they were arrested. The cells were tested for their ability to express lactogenic hormone receptors and to respond to lactogenic hormones by transcribing milk protein genes, as did primary mammary cells grown on floating collagen gels (10, 28). In transformed cells, there was a progressive loss with time in cell surface expression of lactogenic hormone receptors. Even at early passages the binding capacity of these cells remained low, and thus it was not possible to estimate the number of receptors. It was therefore not surprising that caseins were not expressed in response to prolactin stimulation. In the immortalized cells, however, the number of receptors was twice that in the primary cells from which they were derived. Despite the presence of a large number of receptors, no hormonally regulated milk protein transcripts were detectable by our assay, which should have allowed us to see a level of specific mRNA at least 10^4 times lower than that of lactating cells. Using Northern blots, we were unable to detect accumulation of casein-specific mRNA. At present we do not know whether casein genes are altered, whether the prolactin-receptor complex is nonfunctional, or whether culture conditions and hormone requirements are different from those for primary mammary cells (28). We intend to introduce the milk protein genomic sequences into these cell lines and test whether they are transcribed and whether transcription is regulated by lactogenic hormones.

The data presented in this paper demonstrate that the assay system used is suitable for studying the immortalizing and transforming potential of known and candidate oncogenes for epithelial cells, particularly oncogenes involved in mammary tumorigenesis. There is evidence that mouse mammary tumor virus can induce mammary tumors in mice by insertional mutagenesis (20, 22; M. Garcia, R. Wellinger, A. Vessaz, and H. Diggelmann, EMBO J., in press), leading to the activation of chromosomal loci. These highly conserved chromosomal loci are potentially new oncogenes involved in epithelial cell transformation. The microinjection assay presented here will be used to test this hypothesis.

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