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CHEMICAL CARCINOGEN-MOUSE MAMMARY TUMOR VIRUS INTERACTIONS IN CELL TRANSFORMATION

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SUMMARY

We have studied the process of mammary cell transformation in vitro using a single cell clone (Clone 18) from a presumptive epithelial cell line, C57MG, derived from a normal mammary gland; a mouse mammary tumor virus (MMTV) host-range variant (RIII)vp4; and the potent initiating carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA). After several serial subcultures, cells treated with virus and then with carcinogen exhibited an altered (transformed) morphology, a dramatic increase in anchorage independence, an increase in multinucleation after exposure to cytochalasin B, an enhanced ability to proliferate in low Ca^{2+} (0.01 m*M*) medium, and tumorigenicity when inoculated subcutaneously into athymic (nude) mice. Although some of these phenotypic alterations were observed also in cultures treated singly with MMTV or DMBA and in cultures exposed to DMBA before infection with MMTV, enhanced cytochalasin B multinucleation and tumorigenicity were properties observed only in mass cultures of cloned cells first infected with MMTV and then exposed to DMBA. This demonstrates for the first time that exposure of presumptive mammary epithelial cells to MMTV followed by DMBA, but not to either agent alone or to DMBA followed by MMTV, results in malignant transformation of these cells.

Key words: MMTV; DMBA; presumptive mammary epithelial cell cultures; in vitro transformation.

INTRODUCTION

Mammary carcinogenesis in mice seems to be a complex process involving the initiation and progression of preneoplastic lesions and is influenced by a variety of agents including ionizing radiation, hormones, chemical carcinogens, and viruses (1–5). Although both chemical carcinogens and mouse mammary tumor viruses (MMTVs) are able to induce preneoplastic lesions in mice, the efficiency of these induction processes can be altered by changing the hormonal balance of the animals (6,7). In addition, other factors, such as stress, diet, and genetics, may play a role in the etiology of murine mammary cancer.

There is now ample evidence that chemical carcinogens and both DNA tumor viruses (8–11) and RNA tumor viruses (12–15) can act in a synergistic fashion. It has been suggested that chemical carcinogens exert their effects through an interaction with cellular macromolecules and that such interactions result in an alteration of gene expression (16,17). The widespread association of type C RNA tumor virus genetic activity with chemically induced transformation (18–20) has led to speculation that one mechanism of transformation by chemical carcinogens may be to act by derepressing oncogenic viruses. It has been reported also, however, that chemical carcinogens may cause transformation in the absence of detectable virus production (21). The synergistic mechanism

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of transformation by viruses and chemicals remains to be elucidated in many systems.

Although attempts have been made to study synergistic interactions between the potent initiating carcinogen DMBA and MMTV in tumorigenesis in the intact animal (22), these studies are difficult to interpret on a cellular level because of the complex intermediate events between administration of the carcinogen and the appearance of tumors, and because of the possible influence of host factors on the transformation process. In the series of experiments reported here, we have developed a cell culture model system to study the interaction of the carcinogen DMBA and MMTV with murine mammary presumptive epithelial cells. We show that treatment of normal mammary presumptive epithelial cells with both DMBA and MMTV results in transformation of the cells as indicated by an increased tumorigenicity in athymic (nude) mice, an increased ability of cells to grow in semisolid medium, an increase in multinucleation after exposure to cytochalasin B, an enhanced ability to proliferate in low Ca^{2+} (0.01 m*M*) medium, and the appearance of discernable morphological changes in cell populations. The results indicate a possible cocarcinogenic interaction between DMBA and MMTV resulting in malignant transformation of the cells.

Materials and Methods

Cells. The C57BL/6 mammary gland cell line, C57MG (23), was obtained from Dr. E. Lasfargues, Institute for Medical Research, Camden, NJ. This line was cloned at Passage 31. Transformation studies were conducted with a single cell clone, designated C57MG (Clone 18). Cells were grown in Eagle's minimal essential medium containing 10% (vol/vol) heat inactivated fetal bovine serum, 10 μg/ml bovine insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. They were passaged at confluence using 0.1% trypsin containing 0.5 m*M* EDTA. Cells were periodically monitored for the presence of mycoplasma and found to be negative. The murine origin of the C57MG cell line was verified by karyotyping, isoenzyme analyses, and immunofluorescence analyses using species specific antisera. The presumptive epithelial origin of the cells was demonstrated by the presence of desmosomes, tonofibrils, and secretory granules (performed by Dr. C. M. Calberg-Bacq, University of Liege, Belgium). The C57MG (Clone 18) cells were incapable of inducing tumors when 2×10^6 cells were inoculated subcutaneously into syngeneic C57Bl/6 or athymic (nude) mice. Because the most stringent criterion presently available for determining if a cell culture system is "normal" is its inability to induce tumors when inoculated into a susceptible host, C57MG (Clone 18) cells are referred to as normal.

Virus and infection of cells. The MMTV(RIII)vp4 was isolated by four serial virus passages in feline CrFK cells as described previously (24). Virions obtained from 24 h collections of cell culture supernatant fluids from infected CrFK cells were concentrated by isopycnic gradient centrifugation in sucrose gradients as described previously (25). Several preparations of virus were pooled and filtered through a 0.45 μm Millipore filter, precoated with polyvinylpyrrolidone (26) to remove any contaminating bacteria. The amount of virus in these stock solutions was determined by group specific radioimmunoassays for the 52,000d major external glycoprotein (gp52) and the 28,000d major internal protein (p28) of MMTVs as described previously (27,28). Aliquots of virus were then stored over liquid nitrogen to provide a stock of virus for reproducible infection of cells.

The procedure for infecting cells with MMTVs has been described previously in detail (29,30). Briefly, the procedure was as follows: cells were seeded in 75 cm^2 flasks at known cell densities and virus was added 24 h later in medium containing 4 μg/ml polybrene. Twenty-four or forty-eight hours later, the medium was changed to medium without polybrene. At appropriate intervals thereafter, 24 h collections of culture medium were assayed for the presence of progeny virus using a group specific radioimmunoassay for MMTV gp52 as described previously (27). When virus, carcinogen + virus, and virus + carcinogen treated cultures were assayed at various times posttreatment, no significant difference was found in the time of virus production (3 to 7 d) or the final level of released virus.

Toxicity of DMBA. To determine the concentration of DMBA to be used in virus-chemical cocarcinogenesis experiments, the toxicity of various concentrations of DMBA for C57MG (Clone 18) cells was measured. Approximately 2×10^6 C57MG (Clone 18) cells were seeded in 100 mm cell culture plates and treated 24 h later with 0.0005 to 5 μg/ml DMBA in 0.1% dimethylsulfoxide (DMSO).

After treatment for 24 h, the carcinogen containing medium was removed and the cells were

washed in sterile phosphate buffered saline (PBS). Control cultures were treated with either 0.1% DMSO (carcinogen solvent) or with growth medium for 24 h. The cultures were then trypsinized and 3×10^2 cells were plated in each of five 100 mm cell culture plates to determine their plating efficiency. Cultures were refed twice per week with fresh growth medium and stained with Giemsa after 10 to 14 d. The number of colonies was then counted. A level of 0.3 μg/ml DMBA was chosen for studies reported in this paper. At this carcinogen concentration the cloning efficiency of treated cells was reduced approximately 20%.

Virus and chemical treatment of cells. Transformation assays were performed as follows: 10^5 C57MG (Clone 18) cells were seeded in 75-cm^2 culture flasks and 24 h later were either mock infected or infected with MMTV(RIII)vp4 at a multiplicity of infection (M.O.I.) of 10^4 particles/cell; 48 h postinfection the cells were treated for 24 h with 0.1% DMSO or 0.3 μg/ml DMBA. Cultures were then washed twice with sterile PBS and refed with fresh growth medium; alternatively, cultures were first treated for 24 h with DMBA, followed by infection with MMTV(RIII)vp4. At confluency cultures were passaged 1:10. The experimental protocol just described resulted in five groups for each clone: Control (mock infected and DMSO treated); virus (MMTV infected and DMSO treated); DMBA (mock infected and DMBA treated); DMBA + virus (DMBA treated and then MMTV infected); and virus + DMBA (MMTV infected and then DMBA treated).

Transformation assays. To determine the tumorigenic potential of cultures in vivo, cells were inoculated subcutaneously at a concentration of 2×10^6 cells in 0.2 ml of serum-free medium. Animals were examined weekly for the appearance of tumors.

The ability of cells to grow in semisolid agar was also determined. Cells were suspended in 0.4% Difco bacto agar (Difco Laboratories, Detroit, MI) in growth medium and 5×10^4 cells in this suspension were overlaid onto 5 ml of 0.8% agar in 60-mm cell culture plates. Cultures were incubated in a humidified incubator at 37° C in an atmosphere of 5% CO_2 for 14 to 21 d. Colonies of diameter greater than 0.2 mm were counted.

Cytochalasin B multinucleation was determined as described by Medina et al. (31,32). Cells were seeded at a density of 1×10^4 cells/cm^2, and 1 μg/ml cytochalasin B in 0.1% DMSO was added 24 h later. After an additional 48 h cells were fixed, stained with Giemsa, and the number of nuclei in a minimum of 300 randomly selected cells was determined. When experiments were performed using different passage number (Passages 10, 12, or 15) cultures, the degree of variation between parallel cultures was ≤5%.

The ability of C57MG (Clone 18) cells to proliferate in medium containing reduced levels of free extracellular calcium was determined as follows. Medium containing 0.01 m*M* Ca^{2+} was prepared by adding the calcium chelater [ethylene bis(oxyethelenenitrilo)] tetraacetic acid (EGTA) to medium prepared without calcium and containing 10% fetal bovine serum (FBS). The amount of EGTA added to complex the serum calcium was determined by analyzing the calcium content of the FBS by atomic absorption spectrophotometry (33). Calcium was then added to the medium using a stock solution of $CaCl_2$ resulting in a final free calcium level of 0.01 m*M*.

Confluent monolayers of cells were trypsinized and seeded at a density of 1×10^3 cells/100 mm petri dish in growth medium containing 0.01 m*M* Ca^{2+}. The medium was changed twice per week until cell colonies were visible with the naked eye. Cells were then fixed, stained with Giemsa, and the number of colonies in each petri dish was determined.

RESULTS

Cytochalasin B multinucleation. At present there are few reliable in vitro assays for differentiating between normal and transformed epithelial cells (34–36). Recent studies by Medina and coworkers (31,32) have indicated that cytochalasin B induced multinucleation can be used to distinguish between normal and malignant murine mammary epithelial cells. As shown in Table 1, normal mammary gland epithelial cells of mouse or rat origin exhibit less than 11% multinucleation when treated with cytochalasin B. In contrast, a mouse mammary tumor cell line displayed greater than 52% multinucleation in the presence of cytochalasin B. When tested after 10 serial subcultures (Passage 10), only virus + DMBA treated C57MG (Clone 18) cells scored as positive for the transformed phenotype, i.e. greater than 30% multinucleation (31,32) (Table 1). Similar results were found also when the five groups of C57MG (Clone 18) cells were

TABLE 1

CYTOCHALASIN B TREATMENT OF NORMAL AND TRANSFORMED CELLS

Cell Line[a]	Origin	Cells With >2 Nuclei[b]
		%
C57MG	Normal mouse mammary gland	11
ACI-MG	Normal ACI rat mammary gland	3
W/L-MG	Normal Wistar-Lewis rat mammary gland	4
Mm5mt/c_1	Murine (C3H) mammary tumor cell line	52
C57MG (Clone 18) cells[c]		
Control	Treated with 0.1% DMSO	15
Virus	Infected with MMTV	18
DMBA	Treated with 0.3 μg/ml DMBA	15
DMBA + virus	Treated with 0.3 μg/ml DMBA and subsequently infected with MMTV	22
Virus + DMBA	Infected with MMTV and subsequently treated with 0.3 μg/ml DMBA	35

[a] The sources of the cells were as follows: ACI-MG and W/L-MG, established by Dr. D. K. Howard; Mm5mt/c_1, Dr. D. Fine, Frederick Cancer Research Center, Frederick, Md.

[b] Cultures with 30% or more of the cells having greater than two nuclei are considered transformed.

[c] Cultures were assayed at Passage 10 posttreatment.

tested after 15 (Table 2) or 18 passages (data not shown).

Proliferation in low calcium medium. Recent studies have demonstrated the importance of Ca^{2+} in regulating the proliferation of normal fibroblast and epithelial cells (37-40). Normal cells from a variety of species are unable to sustain growth when the extracellular Ca^{2+} level is less than 0.02 m*M*, whereas their chemical or viral transformed counterparts proliferate even when the level of extracellular Ca^{2+} is as low as 0.001 m*M* (39-42). As shown in Table 2, DMBA, DMBA + virus, and virus + DMBA treated C57MG (Clone 18) cells were capable of proliferating in

TABLE 2

COMPARISON OF MARKERS OF TRANSFORMATION IN VIRUS, CHEMICAL AND COMBINATION TREATED C57MG (CLONE 18) CELLS

Cell Type	Morphology[a]	Growth in Low Ca^{2+} Medium[b]	Cytochalasin B Multinucleation[c]	Growth in Agar[d]	Nude Mice[e]
		%	%	%	
Control	N	3	17	0.07±0.05	0/4
MMTV	N	2	21	0.06±0.03	0/4
DMBA	T	6	20	0.55±0.18	0/4
DMBA + MMTV	T	5	21	0.4 ±0.09	0/4
MMTV + DMBA	T	8	35	1.1 ±0.08	4/4
Subclones of MMTV+DMBA treated Clone 18 cells[f]					
MMTV + DMBA, Subclone 1	N	1	18	0.48±0.01	5/5[g]
MMTV + DMBA, Subclone 3	T	7	32	0.26±0.02	5/5
MMTV + DMBA, Subclone 4	T	4	14	0.01±0.0	5/5

[a] N = Normal (contact inhibited and well defined borders); T = transformed (random orientation and the appearance of numerous long, thin pseudopodia). This represents the predominant morphology of cells.

[b] Cultures were tested at Passage 15; control, MMTV and MMTV+DMBA (Subclone 1) produced small colonies <25 cells in 0.01 m*M* Ca^{2+}.

[c] Cultures containing ⩾30% cells containing greater than two nuclei per cell were considered transformed. Cultures were tested at Passage 15.

[d] Growth in soft agar was determined as described in Materials and Methods.

[e] Twelfth passage cells were inoculated subcutaneously in female BALB/c athymic (nude) mice at a concentration of 2×10^6 cells in 0.2 ml of serum-free medium. Animals were observed for the appearance of tumors for a period of 12 to 14 wk.

[f] Single cell clones (subclones) were isolated from 1st passage cultures infected with MMTV and treated for 24 h with 0.3 μg/ml DMBA 48 h postinfection.

[g] Small tumors appeared 12 wk after inoculation of cells, but regressed 3 wk later.

0.01 m*M* Ca^{2+} containing medium (large colonies, >3% cloning efficiency). This was not the case, however, with control or virus treated Clone 18 cells, which exhibited only marginal growth in low Ca^{2+} medium (small colonies, <3% cloning efficiency). All of the Clone 18 cultures that grew in low Ca^{2+} medium also displayed a predominantly transformed morphology (Fig. 1, Table 2).

Growth in semisolid agar. An in vitro property that is often found to correlate with in vivo tumorigenicity of transformed fibroblast and some transformed epithelial cells is anchorage independence, i.e. the ability of cells to grow when suspended in agar, agarose, or methylcellulose (35,43). In the present study, DMSO treated C57MG (Clone 18) cells formed macroscopic colonies in agar with a frequency of less than 0.01%, when seeded in agar, whereas virus + DMBA treated cells exhibited a greater than 100-fold enhancement in anchorage independence (Table 2). A smaller increase in cloning efficiency

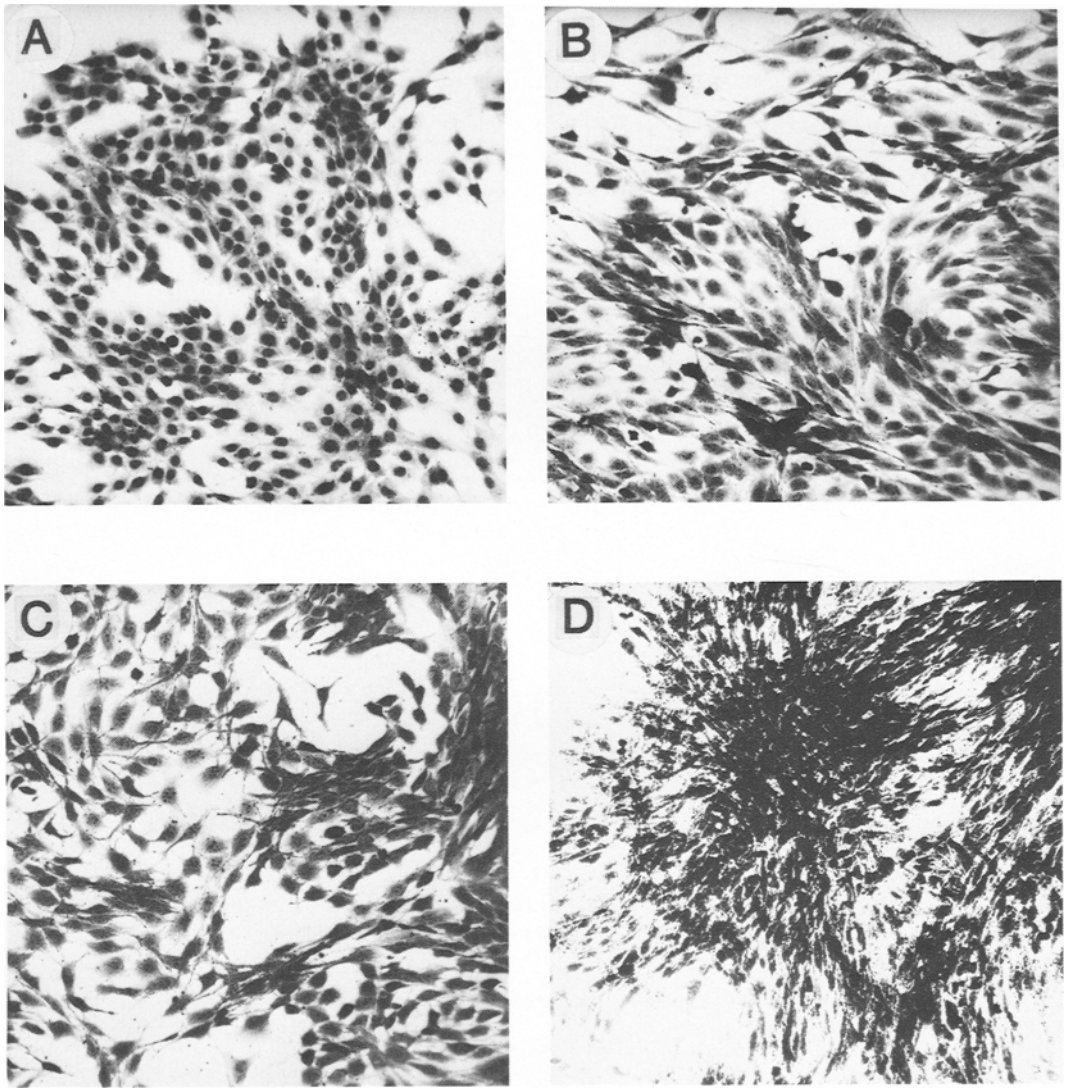

FIG. 1. Photomicrographs of stained C57MG (Clone 18) cells and virus, carcinogen, and virus + carcinogen treated Clone 18 cells. Approximate ×90. Control and treated cultures were passaged 18 times before photographing. *A*, control (0.1% DMSO). *B*, MMTV infected. *C*, DMBA (0.3 μg/ml) treated. *D*, MMTV infected and subsequently treated with 0.3 μg/ml DMBA.

in agar was observed in DMBA, and in DMBA + virus treated Clone 18 cells. In contrast, cells infected with MMTV formed colonies in agar with the same low efficiency as control cells.

Tumorigenicity in nude mice. It is now generally agreed that the carcinogenic process often proceeds through several stages in its development (35,43,44) and that a later step in this process is the appearance of the malignant phenotype. The ultimate test for transformation is still the ability of cells to grow as tumors when injected into syngeneic or athymic (nude) mice. Only virus + DMBA treated C57MG (Clone 18) cells induced tumors at the site of inoculation in nude mice (Table 2). A similar trend has been observed in another single cell clone of C57MG cells, Clone 2 (Table 3). With both Clones 18 and 2, tumorigenicity was not observed until the MMTV + DMBA treated cells had been passaged serially in culture for a minimum of five times (Tables 2 and 3). Heterogeneity within the treated mass cultures is to be expected and was demonstrated by subcloning the virus + DMBA treated Clone 18 cells and analyzing the properties of these clones (Table 2). Only subclone three of the virus + DMBA treated Clone 18 cells was positive in all five of the tests, whereas Subclone 1 was normal by the five criteria and Subclone 4 was positive in three of the five tests (Table 2). A similar heterogeneity was observed in subclones of virus, DMBA, and DMBA + virus treated Clone 18 cultures (unpublished data). By analyzing a series of subclones from the various groups of treated C57MG (Clone 18) cells it should be possible to determine which in vitro phenotype directly correlates with in vivo tumorigenicity.

TABLE 3

TUMORIGENICITY OF C57MG (CLONE 2) MAMMARY GLAND CELLS IN ATHYMIC (NUDE) MICE[a]

Passage Level After Treatment	Number of Animals With Tumors/Total Number Animals			
	Control	Virus	DMBA	Virus + DMBA
1	0/20	0/20	0/20	0/20
3	0/8	0/8	0/8	0/8
5	1/16	1/16	0/16	6/16
7	0/8	0/8	0/8	3/8
Passages 5 to 7	1/24	1/24	0/24	9/24

[a] Clone 2 cells were isolated from the C57MG cell line. Cells were treated with various combinations of DMBA (1 μg/ml) and MMTV. At every 2nd passage after treatment, cells were inoculated into athymic (nude) mide. Cells were inoculated subcutaneously at a concentration of 2×10^6 cells in 0.2 ml of serum-free medium. The animals were observed for the appearance of tumors for a period of 12 to 14 wk.

DISCUSSION

The data presented in this paper indicate that a clonal population of mammary derived presumptive epithelial C57MG (Clone 18) cells can be converted to a malignant phenotype after combined treatment with MMTV followed by a carcinogen. In contrast, treatment of these cells with the virus or carcinogen alone, or the carcinogen followed by the virus, does not result in malignant transformation. A similar temporal relationship has been observed in transformation of rat and mouse embryo cells by the combination of murine leukemia virus and various carcinogens (12,14,45,46). Transformation was only observed when cultures were first infected with the murine leukemia virus and then exposed at a later time to the carcinogen (12,14,45,46). Although the mechanism by which the combined viral-chemical treatment results in transformation is not known, it is tempting to try to relate these observations to what is presently known about multiple interactions in mouse skin carcinogenesis (47). Studies on mouse skin have demonstrated two distinct phases, one termed "initiation" and the other "promotion." Initiators are not tumorigenic when applied singly, but when followed with a promoting agent multiple skin neoplasms develop (47). Using this model, MMTV would be functioning as a weak initiating agent. It is worth noting, therefore, that of the four experimental groups cultures infected with MMTV resemble most closely the phenotype of the DMSO controls. The subsequent stage in transformation of the appropriate target cell results when the activated metabolite of DMBA interacts with a specific subset of infected cells.

The delay in the onset of expression of tumorigenicity might result because the original culture is heterogeneous and, therefore, requires several passages to allow for selective outgrowth of tumorigenic cells. Heterogeneity in the treated cultures is expected inasmuch as it is extremely unlikely that every cell infected with MMTV also interacted with the carcinogen, resulting in transformation.

The lack of immediate expression of the complete transformed phenotype, i.e. tumorigenicity, after infection with MMTV and subsequent treatment with DMBA, might also result because a subset of transformed cells must undergo further

progressive changes before acquiring the malignant phenotype (43,48,49). In the case of benzo(*a*)pyrene treated Syrian hamster cells, transformation involves a series of progressive alterations that occur in a well-defined temporal sequence. Morphological transformation is observed by 8 d posttreatment and fibrinolytic activity is expressed by 14 d posttreatment, whereas anchorage independence and tumorigenicity are not manifested until 6 or more weeks after exposure to the carcinogen (48,50). Progressive alterations in expression of the transformed phenotype after repeated subculture have been observed also in Type 5 adenovirus transformed rat embryo (RE) cells (11,49,51), herpes simplex virus Type 2 transformed RE cells (52), feline sarcoma virus transformed human diploid fibroblasts (53), and murine sarcoma virus transformed murine fibroblasts (51). The altered morphology and ability of DMBA and DMBA + virus treated Clone 18 cultures to grow in low Ca^{2+} medium, but not multinucleate in the presence of cytochalasin B or induce tumors in nude mice, may indicate that these cells have acquired early markers of transformation (Table 2). By subcloning the original treated cultures it should be possible to generate a series of cell strains that exhibit only a partial expression of the transformed phenotype. These cells will prove useful in defining the temporal sequence of phenotypic alterations involved in progression of transformation in mammary epithelial cells. The presently described transformation system, in conjunction with currently available techniques for probing gene structure and function, may now help in defining, on a molecular level, the roles of multiple agents (viruses, chemical carcinogens, hormones, tumor promoters, growth factors, and others) in the initiation and promotion of mammary carcinogenesis.

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