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ESTABLISHMENT AND CHARACTERIZATION OF IMMORTALIZED NON-TRANSPLANTABLE MOUSE MAMMARY CELL LINES CLONED FROM A MMTV-INDUCED TUMOR CELL LINE CULTURED FOR A LONG DURATION

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ABSTRACT

During the culturing of a mouse mammary tumor cell line, MuMT73, maintained *in vitro* for more than a decade, we found morphological heterogeneity in its cells; some showed contact inhibition in their growth, some formed domes and some grew criss cross and piled up. In trying to clone the cell line to isolate cells showing contact inhibition or dome formation, we were able to establish six clonal cell lines. These six cell lines were categorically divided into three groups according to their phenotypical behavior, Groups A, B and C. Group A (clones 1, 5 and 7) cells had a property of contact inhibition. They induced no tumor when injected into the subcutaneous tissue of the back, nor even when injected into the mammary fat pads or under the kidney capsule of syngenic or nude mice, and therefore were thought to be non-malignant in nature. They were positively stained by anti-keratin antiserum and had mouse mammary tumor viruses (MMTVs). Group B (clone 6) cells grew in a crisscross pattern and piled up, and they induced tumors when injected into the subcutaneous tissue of the back of mice. Group C (clones 3 and 4) cells formed domes in their growth and induced some tumors in the mammary fat pads and under the kidney capsule of KSN nude mice. In Southern blots with MMTV-*env* probe, numerous exogenous MMTV proviruses were detected in these cell lines. The insertion patterns of these proviruses in cells of non-malignant clonal lines (Group A) resembled those of malignant lines (Group B), except one band (about 26 Kb), but were considerably different from those of intermediate lines (Group C). On the other hand, no difference was detected in Southern blots with *int*-1 or *int*-2 probes among the non-malignant, intermediate and malignant clonal cell lines.

Key Words: A MMTV-induced tumor cell line, Contact inhibition, Non-transplantable cell lines, Oncogenes.

INTRODUCTION

Foulds and others have reported the multistage model of neoplastic progression since 1958.[1,2] Most investigators assumed that naturally occurring tumors were the result of a series of somatic cell mutations, each providing the neoplastic cell with further selective growth advantage.[3] In this sense, non-transplantable mammary cell lines are thought to be important for

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the study of mammary carcinogenesis. Many mammary tumor cell lines have been reported,[4,5] but few lines have been described as normal mammary cell lines.[6]

When we cultured a mouse mammary tumor cell line, MuMT73, we found severe heterogeneities in its cells; some showed contact inhibition in their growth, some formed domes, and some grew in a crisscross pattern. We tried to isolate clonal cell lines with different morphologies from this cell line, and were able to establish six lines. These cell lines are thought to be useful for studying mammary carcinogenesis. Therefore, we characterized phenotypical behavior as well as genetic behavior in these clonal cell lines. We also examined proviral insertion of mouse mammary tumor virus (MMTV) and activation of putative oncogenes *int* by Southern blotting, since MMTV is known to lead to a high incidence of mammary neoplasia,[7] by proviral insertion which often activates cellular oncogenes, such as *int*-1 and *int*-2.[8,9]

MATERIALS AND METHODS

Cell Culture: A mouse mammary tumor cell line, MuMT73, was established from spontaneous mammary tumors of BALB/cfC3H mice by Sarkar *et al.*[10] in 1973. This cell line has been maintained in our department since 1984. Cells were cultured in a growth medium of Dulbecco modified Eagle medium containing 10% fetal bovine serum (FBS).

For cloning of the MuMT-73 cell line, trypsinized cells were plated into 96-well tissue culture plates to give cell concentrations of 1 cell/well by limiting dilution. Six clonal cell lines were established and named as clones S1, S3, S4, S5, S6 and S7. Clones S1, S5 and S7 were subcultured in a split ratio of 1 to 3 once a week, and clones S3, S4 and S6 were subcultured in a 1:10 ratio once a week.

Colony Forming Rate (CFR) in Soft Agar: Trypsinized cells were resuspended in 0.3% agar (Difco, Detroit) in the growth medium at a concentration of either 5.0×10^3 for clones S3, S4 and S6 respectively, and 5.0×10^4 cells/ml for clones S1, S5 and S7, respectively. A half ml of the cell suspension was overlaid on a half ml base of 0.6% agar in the growth medium in 24-well tissue culture plates. Two weeks after plating, the CFR in soft agar was determined by counting the colonies in five fields in duplicate wells on a phase contrast microscope, and total numbers in each well were finally estimated. To determine the CFR on plate, cells (1×10^3) were dispersed into 25 cm^2 plastic culture flasks. Two weeks after plating, the CFR on plate was determined by counting the numbers of colonies in the flasks.

Transplantability in Mice: Trypsinized cells were washed three times with serum free medium. Cells (1×10^5 to 1×10^7) were injected into the subcutaneous tissue of the back, the mammary fat pads or the kidney capsule of 4- to 6-week-old female BALB/c mice, BALB/c nude or KSN nude mice. The mice were observed weekly for more than three months for the appearance of tumors.

Electron Microscopic Study: Tripsinized cells were washed three times with phosphate buffered saline (PBS) and were fixed with 2.5% glutaraldehyde-paraformaldehyde in 0.1 M PBS (GA-PA) followed by 1% OsO_4 in the same buffer, then stained with uranium, dehydrated with graded ethanols (EtOH) at room temperature or at 4°C and embedded in quetol. Ultrathin sections were stained with uranium and lead, and observed with a transmission electron microscope, Hitachi H-600, at 75 KV.

DNA Synthesis at a Confluent State: When cells reached confluency on coverslips, [^{3}H]thymidine (10 μCi/ml) was added to the culture medium. After 16 hr the coverslips were washed three times with PBS, and fixed in Carnoy's solution (60% ethyl alcohol, 30% chloroform and 10% acetic acid) for 20 min. The coverslips were then covered with Sakura autoradiographic

emulsion and exposed thereto in the dark for ten days. After development, the preparations were stained with hematoxylin and eosin (H-E) and examined microscopically.

Antisera: Rabbit antiserum to bovine keratin and rabbit antiserum to chicken desmin were purchased from DAKO, Santa Barbara and Bio-Science, Emmerenbrucke, Switzerland. Rabbit antiserum to MMTV[11] and rabbit antiserum to mouse plasma fibronectin[12] were prepared as previously described. Rabbit antiserum to mouse laminin was a gift from Dr. K. Kimata,[13] Nagoya University School of Science. Rat monoclonal antibodies to mouse mammary epithelial cells (33A10 and JsE3) were gifts from Dr. J. Hilgers[14] of the Netherlands Cancer Institute. Rhodamine-labeled phalloidin was purchased from Molecular Probes, Inc., Junction City, OR. Rhodamine-labeled goat anti rabbit IgG and FITC-labeled goat anti rat IgG were purchased from Cappel Laboratories, West Chester, PA, and Boehlinger Mannheim Biochemicals, Indianapolis, IN, respectively.

Immunofluorescence: Indirect immunofluorescence was performed by the method described previously, using cells cultured on coverslips.[14] For staining keratin filaments, the cells were fixed in 95% ethanol, 1% acetic acid in PBS on ice for 60 min. To stain actin filaments, MMTV antigen, laminin, fibronectin and desmin, the cells were fixed in 4% paraformaldehyde PBS solution, containing 5% sucrose, 0.5mM Ca^{++} and Mg^{++}, for 30 min and then treated with 0.2% triton-X 100 in PBS for 4 min. For staining with monoclonal antibodies, 33A10 and JsE3, cells were treated in cold acetone for 2 min and air-dried. These preparations were examined with an Olympus fluorescent microscope with suitable filters for each fluorescent dye.

Southern Blot Analysis: Cells of each cell line were cultured in ten plates of plastic dishes (100 mm in diameter). Upon reaching confluency, cells were washed with PBS, scraped off with a rubber policeman, centrifuged and resuspended in 10 ml of 500 mM EDTA, 0.5% sodium sarcosinate, 1 mg/ml of proteinase K. After the mixture was incubated overnight at 37°C, DNA was extracted twice with phenol, twice with phenol-chloroform (1:1) and once with chloroform according to the method recommended by Maniatis.[15] Ethanol-precipitated DNA was collected by spooling on a glass rod, then by air-drying and dissolving in distilled water. The nucleic acid concentration was determined by measuring the optical density at 260 nm. DNA from liver tissue was used as a reference.

Cellular DNAs (10 μg) were digested in a 4-fold excess of *Eco*RI (Takara Co., Kyoto). The digested DNAs were subjected to electrophoresis in 0.6% agarose gels and transferred to nylon membranes (Gene Screen *Plus* filters; New England Nuclear, Boston) by the method of Southern.[16] Recombinant plasmid pBR322 containing the ectopic specific sequence of either MMTV-*env* or *int*-1,[8] and plasmid pAT154 containing the sequence of *int*-2[9] were used as probes. MMTV-*env* was a gift from Dr. H. Tanaka of Kyoto University. The DNAs (40 ng) were labeled by random-priming in reactions containing 25 μCi each of [alpha-^{32}P]dCTP and multiprime labeling kit (Amersham International Plc., Amersham, U.K.). Membrane-bound DNA fragments were hybridized to [alpha-^{32}P]-labeled probes in a mixture of 50% formamide, 10% dextran sulfate, 1% SDS and 1M NaCl for 18 hr at 42°C. The filters were washed according to the manufacturer's manual of Gene Screen *Plus*, air-dried, and exposed to Kodak XAR-5 films with intensifying screens (Du Pont Co., Boston).

RESULTS

Isolation of Different Clones from an Original Cell Line and their Morphology: We found that the original cell line, after being cultured for a long duration, was made up of three types of cells morphologically different cells: more than half of the cells were growing with a piled up

pattern, some formed domes and others showed contact inhibition at a confluent state. We tried to clone cell lines by limiting dilution to obtain cells with contact inhibition or cells that formed domes. We succeeded in isolating six clonal cell lines.

Clones S1, S5 and S7 formed a flat monolayer of cuboidal cells at a confluent state and showed contact inhibition in their growth (Fig. 1a). Cells of these three clones did not grow with daily changes of the medium containing 1% or 2% FBS. Upon repeated passages at a subconfluent state, they were found to be stable with regard to contact inhibition and many other phenotypes described later, although some cells were observed to pile up upon repetition of more than ten passages at a confluent state. Cells of clone S6 were elongated (Fig. 1b), grew in a crisscross pattern and reached high densities. Cells of clones S3 and S4 were polygonal at a confluent state. When they were allowed to grow further, they packed tightly together and spontaneously formed domes (Fig. 1c).

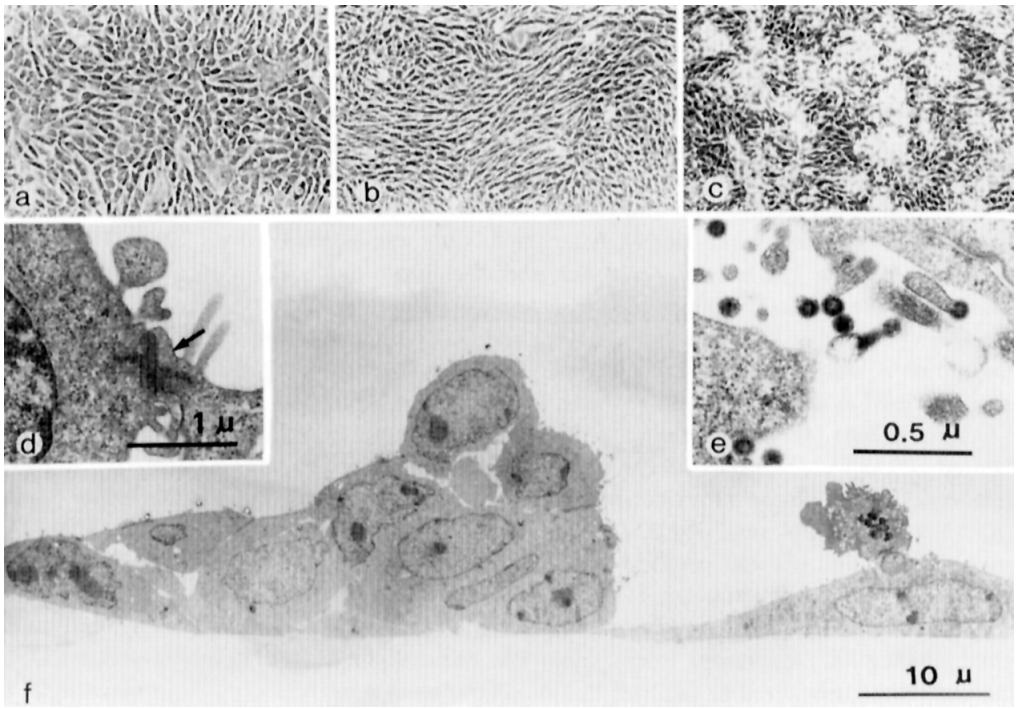

Fig. 1. Phase contrast (a, b, c) and electron microscopic features (d, e, f) of cells of the clonal cell lines. Cells of clone S5 (Group A) form a flat monolayer of cuboidal cells at a confluent state (a). Cells of clone S6 (Group B) are elongated and grow in a crisscross pattern (b). Cells of clone S3 (Group C) are polygonal and form domes (c). Desmosome-like structure (d) and many B-particles of MMTV (e) are seen in cells of clone S3. Dome-like structures (f) are formed by the cells of clone S3.

Doubling Time and Saturation Density: The doubling times were 25 to 26 hr in clones S1, S5 and S7, about 14 hr in clone S6, 17 to 18 hr in clones S3 and S4 (Table I). The doubling times of clones S1, S5 and S7 were notably longer than those of the other clones.

The saturation densities were 0.5 to 0.6 $\times$ 10^5 cells/cm^2 in clones S1, S5 and S7, 4.0 $\times$ 10^5 cells/cm^2 in clone S6, and 1.8 to 2.0 $\times$ 10^5 cells/cm^2 in clones S3 and S4 (Table I). The saturation densities of clones S1, S5 and S7 were notably lower than those of the other clones.

Electron Microscopic Studies: Cells of all clonal cell lines had the general features of epithelial cells, including many desmosomal structures in cells of clones S3 (Fig. 1d) and S4, and some in cells of the other clones. Many B-particles and some C-particles of MMTVs were also found (Fig. 1e).

Colony Forming Rate (CFR) in Soft Agar: Cells of clones S1, S5 and S7 produced no colonies in soft agar, while cells of clone S6 formed many colonies. Cells of clones S3 and S4 were easy to pack together, so colonies formed in soft agar at about 4% in dispersion, and the number of colonies did not increase even after two weeks. The CFR in soft agar was 25% in clone S6. The CFR on plate in all clonal lines was 14.5% to 26.0% (Table I). Neither colony formation of clones S1, S5 and S7 nor increase in the CFR of clones S3 and S4 was induced by adding conditioned medium of clone S6.

Table I. Doubling time, saturation density, CFR[a] in soft agar, CFR on plate, and labeling index of [^{3}H]thymidine of the clonal cell lines

Cell line	Doubling time (hr.)	Saturation density (cells/cm^2)	CFR in soft agar[b] (%)	CFR on plate[c] (%)	Labeling index (%)
S1	26.7	0.6 $\times$ 10^5	0.0	16.2	1.5
S5	26.3	0.5 $\times$ 10^5	0.0	26.0	N.D.[d]
S7	25.7	0.6 $\times$ 10^5	0.0	20.3	N.D.
S6	13.9	4.0 $\times$ 10^5	25.6	15.4	26.8
S3	16.4	2.0 $\times$ 10^5	3.8	20.5	19.0
S4	18.5	1.8 $\times$ 10^5	3.4	14.5	N.D.

[a] Colony forming rate.

[b] Number of colonies in soft agar/number of cells seeded. (Number of cells seeded was 5 $\times$ 10^4 for clones S1, S5 and S7, and 5 $\times$ 10^3 for clones S3, S4 and S6.)

[c] Number of colonies on plate/number of cells seeded. (Number of cells seeded was 1 $\times$ 10^2 for each cell line.)

[d] Not done.

Transplantability in Mice: While cells of the clone S6 induced tumors in the subcutaneous tissue of the back of BALB/c mice or BALB/c nude mice (BALB/c-nu/nu), 10^5 to 10^6 cells of clones S1, S3, S4, S5 and S7 induced no tumors. The histology of the implanted tumors in mice derived from clone S6 were poorly differentiated carcinomas with sarcomatous appearance. Even 10^7 cells of clone S1 induced no tumors in the mammary fat pads or under the kidney capsule of KSN nude mice, although 10^7 cells of clone S3 induced tumors in the mammary fat pads of one out of two KSN nude mice, and under the kidney capsule of a KSN nude mouse

(Table II). The histology of the implanted tumor of clone S3 in the mammary fat pads and under the kidney capsule was poorly differentiated adenocarcinoma showing no sarcomatous appearance like that of clone S6.

Table II. Transplantability in mice

	Subcutaneous tissue of the back			Mammary fat pad	Kidney capsule
Strain	BALB/cA		BALB/c-nu/nu	KSN-nu/nu	KSN-nu/nu
Cells/mouse	10^6	10^5	10^7	10^7	10^7
S1	0/5	0/10	0/3	0/2	0/1
S5	0/5	0/10	0/3	N.D.[a]	N.D.
S7	0/5	0/10	0/3	N.D.	N.D.
S6	5/5	8/10	3/3	N.D.	N.D.
S3	0/5	0/10	0/3	1/2	1/1
S4	0/5	0/10	0/3	N.D.	N.D.

[a] Not done.

DNA Synthesis at a Confluent State: Only 1.5% of the cells of clone S1 incorporated [^{3}H]thymidine for 16 hr at a confluent state, while the labeling indices were 19.0% in clone S3 and 26.8% in clone S6. The index of clone S1 was notably lower than that of the other clones, in good agreement with its property of contact inhibition (Table I).

Expression of MMTV, Cytoskeletal Filaments (Keratin and Actin), Mammary Epithelial Antigens (33A10 and JsE3) and Organization of Extracellular Matrix (Laminin and Fibronectin): Expression of MMTV in clonal cells was examined by an immunofluorescence method with antibody against whole MMTV particles. MMTV antigens were detected as fine granular staining in all clonal cells (Figs. 4a, 4b and 4c). In electron microscopic study on each clonal cell, a lot of budding forms of MMTV and extracellular type B particles (Fig. 1e) were observed on the cell surface, although intracytoplasmic type A particles were very few. The antigen detected by immunofluorescence seemed to correspond to budding virus particles.

In almost all cells of clones S3 and S4, keratin filaments were detected as irregular fine networks throughout the cytoplasm by immunofluorescence with anti-keratin antibody (Fig. 2f), while no staining was detected in other clonal cells (Figs. 2b and 2d). To induce expression of keratin filaments,[17] 10^{-3}M dibutyryl cyclic-AMP or retinoic acid was added to the culture medium at the final concentration for 24 hr, and then the cells were fixed and stained as described. After culturing with dibutyryl cyclic-AMP, fine keratin filaments were detected in some cells of clones S1 and S6 (Figs. 3b and 3d), while they were not detected in any cells cultured without it (Figs. 2b and 2d). And after culturing with retinoic acid, weakly stained keratin filaments were detected in almost all the cells of clones S1 and S6.

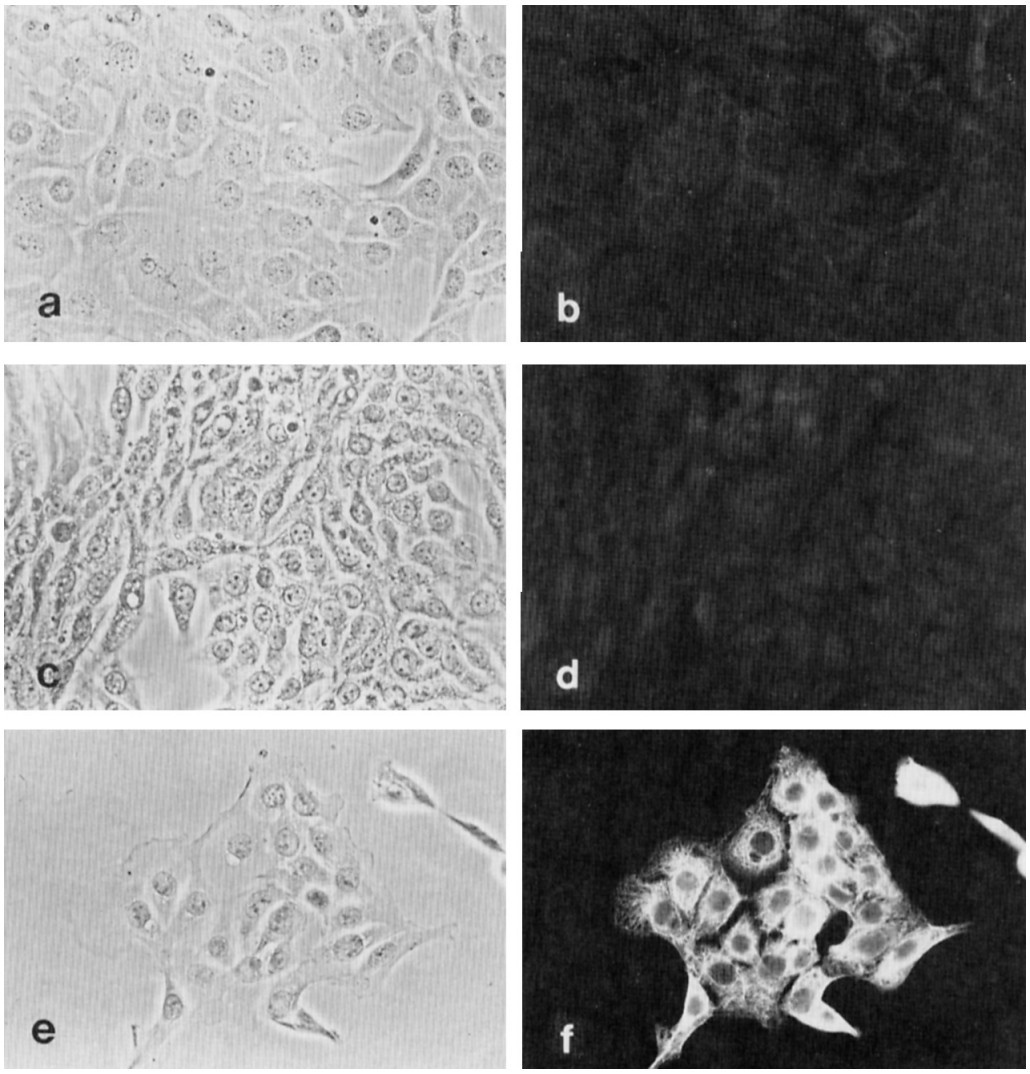

Fig. 2. Phase contrast (a, c, e) and immunofluorescent micrographs (b, d, f) stained with anti-keratin antibody. No staining is detected in the cells of clone S1 (b) (Group A) and S6 (d) (Group B), whereas in the cells of clone S3 (Group C) keratin filaments are detected as irregular fine networks throughout the cytoplasm (f).

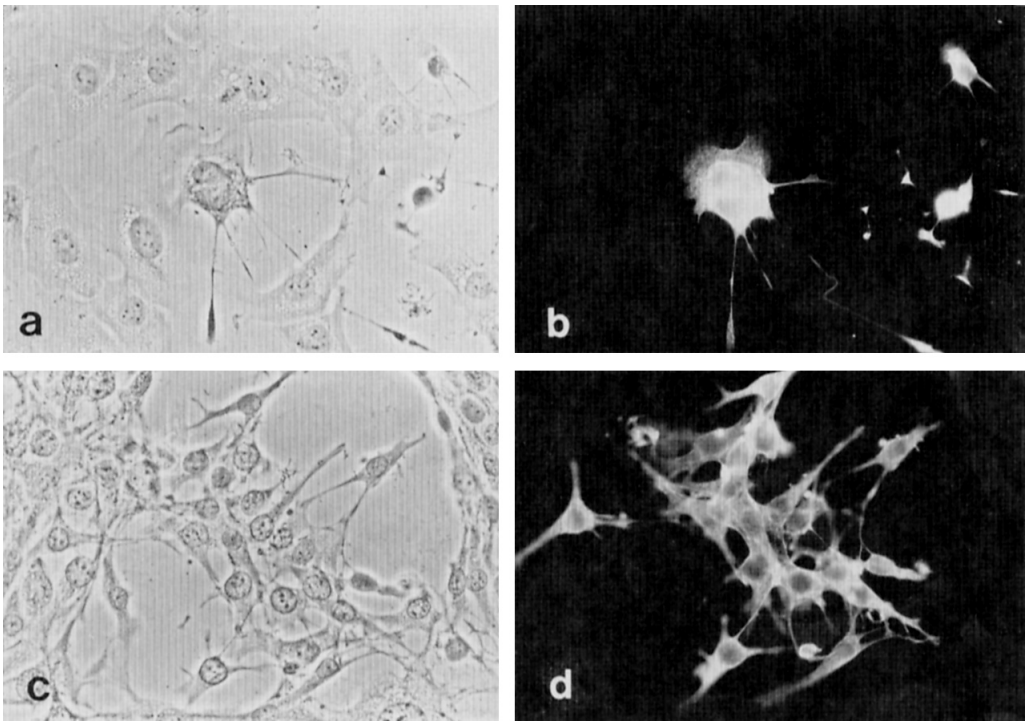

Fig. 3. Phase contrast (a, c) and immunofluorescent micrographs (b, d) stained with anti-keratin antibody after culturing with dibutyryl cyclic-AMP. Fine keratin filaments are detected in some cells of clones S1 (b) and S6 (d), while they are not detected in any cells cultured without dibutyryl cyclic-AMP (Figs. 2b and 2d).

Rat monoclonal antibodies against mouse mammary epithelial antigens, 33A10,[14] reacting with luminal cells of mouse mammary glands, and JsE3,[14] reacting with basal cells of mammary glands, were used in order to decide the origin of clonal cells. 33A10 stained the plasma membrane of the cells in contact with each other of clones S3 and S4, but not that of the dissociated cells, while no staining was detected in other clonal cells (data not shown). In contrast, none of these six clonal cells were stained with JsE3 (data not shown).

Since it is reported that organization of actin filaments in cultured cells is closely related to transformation phenotypes,[18] we examined actin fibers. Actin was clearly demonstrated as fine filament structures in clones S1 (Fig. 4d), S5 and S7, and weakly demonstrated in clones S6 (Fig. 4e), S3 (Fig. 4f) and S4.

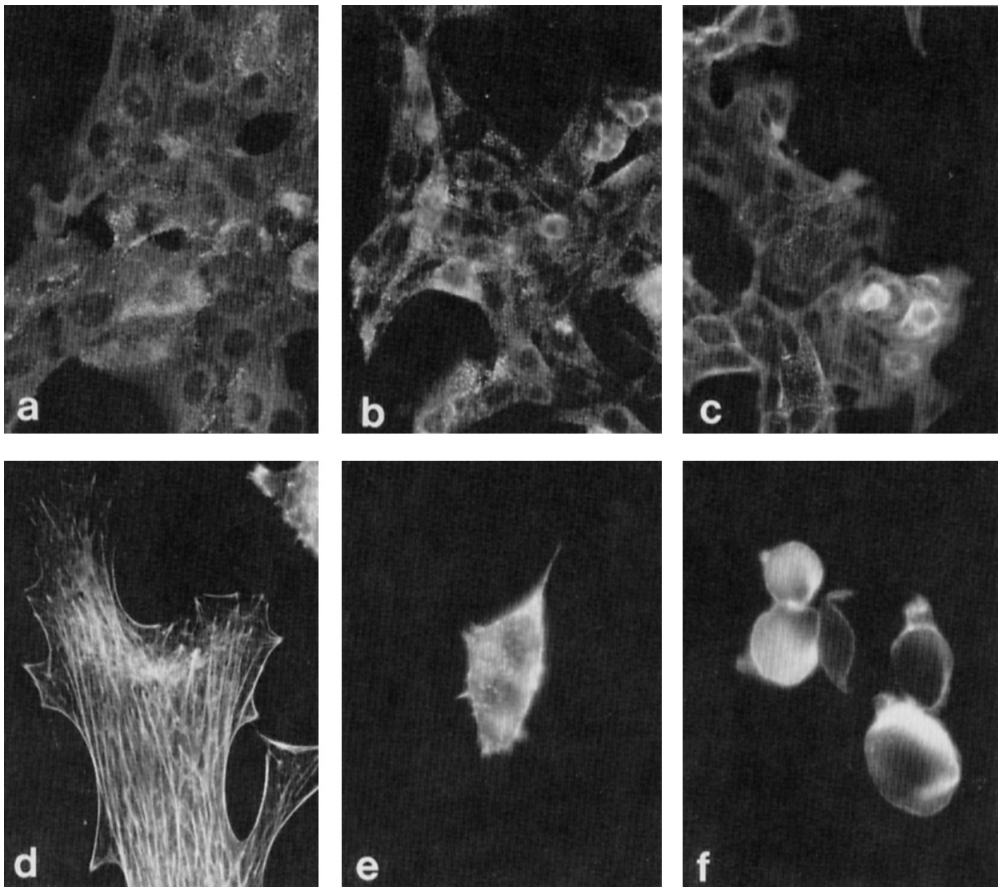

Fig. 4. Immunofluorescent micrographs of cells of the clonal cell lines stained with anti-MMTV antiserum (a, b, c) and with phalloidin (d, e, f). MMTV antigens are detected as fine granular staining in cells of clone S1 (a), S6 (b) and S3 (c). Actin is clearly demonstrated as fine filamentous structures in clone S1 (d) and weakly in clones S6 (e) and S3 (f).

None of these six clonal cell lines reacted with antiserum against desmin, a mesenchymal marker (data not shown). Fibronectin was distributed as a fibrillar network in clones S1, S5, S6 and S7, but could be seen only faintly in clones S3 and S4 (data not shown). Laminin was detected as fine granules arranged along the cell periphery of all clonal cells (data not shown).

Southern Blot Analysis in Clonal Cell Lines: MMTV insertion sites in each clonal cell line with MMTV-*env* were examined by Southern blot analysis. Southern blot analysis of *Eco*RI fragments of DNA from the six clonal cell lines revealed that numerous exogenous MMTV proviruses were inserted in addition to the three endogenous proviruses 16.7, 10.0, and 6.7 kb.

Clones S1 and S6 showed a similar insertion pattern with all three endogenous proviruses, but differed in one band of exogenous proviruses, about 26 kb fragment (triangle in Lane 3 in Fig. 5a). This fragment was seen in clone S6, while not in clone S1. The insertion patterns of the exogenous MMTV proviruses in clones S3 and S4 differed greatly from those of clones S1 and S6, and three endogenous proviruses (16.7, 10.0 and 6.7 kb) were not detected in *Eco*RI fragments of clone S3 (Fig. 5a). The insertion patterns in clones S5 and S7 were the same as that in clone S1.

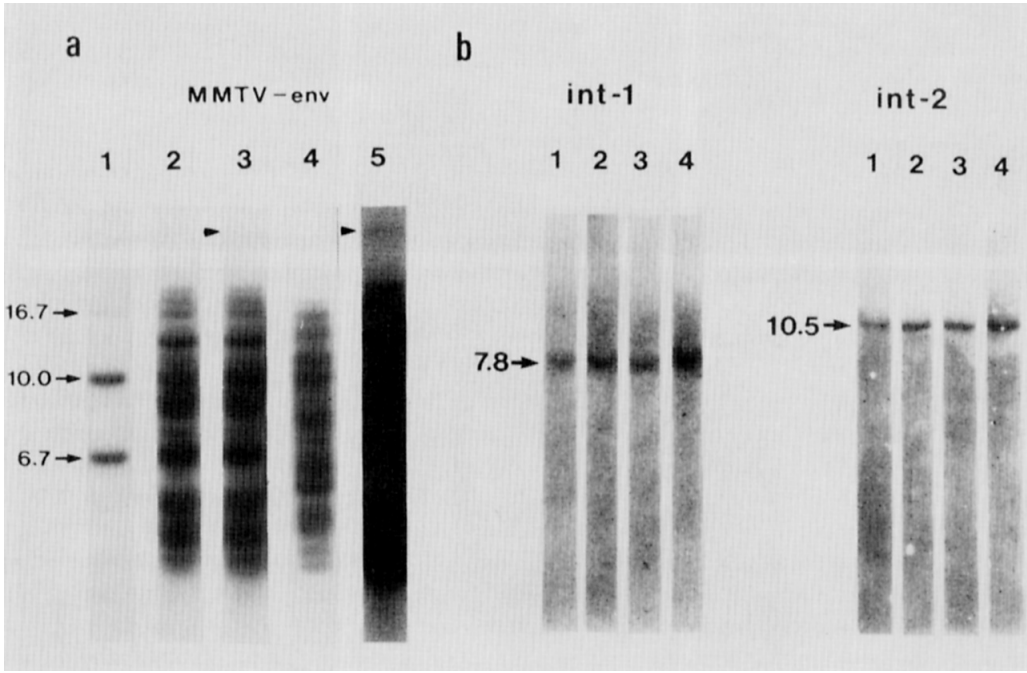

Fig. 5. Southern blots with MMTV-*env*, and *int*-1 and *int*-2. DNA from liver of BALB/c mouse, clone S1, S6 and S3 was digested with *Eco*RI (lane 1, 2, 3 and 4, respectively).
(a) Southern blot with MMTV-*env*. Clone S6 (lane 3 and 5) shows an insertion pattern of three endogenous proviruses, numerous exogenous proviruses, similar to those of clone S1 (lane 2), but associated with one additional band of exogeous proviruses, about 26 kb fragment (triangle in lanes 3 and 5). The insertion patterns in clone S3 (lane 4) were quite different from those of clones S1 and S6. Lane 5 was a long exposed photo which was the same blot as lane 3.
(b) Southern blots with *int*-1 and *int*-2. Only one band of 7.8 kb or 10.5 kb, respectively, was detected with *int*-1 or *int*-2 probes in all cells of clones S1 (lane 2), S6 (lane 3) and S3 (lane 4).

Common integration sites in MMTV proviruses have been reported to be related to mammary carcinogenesis, which led to the identification of putative mammary oncogenes such as *int*-1 and *int*-2. In MMTV-induced mammary tumors proviral activation of *int*-1 or *int*-2 oncogenes is often detected. Thus we examined restriction fragment patterns of *int*-1 and *int*-2 in the clonal cell lines by Southern blot analysis. In all clonal cells only one band of 7.8 kb or 10.5 kb was detected in the Southern blot with *int*-1 or *int*-2 probe, respectively (Fig. 5b).

DISCUSSION

In this paper we have described the properties of a set of six clonal cell lines derived from a spontaneous mouse mammary tumor cell line that was maintained for a long duration. With regard to the morphological features and other phenotypes examined, we divided these six clonal cell lines into three groups (A, B and C), which were distinct in many respects. The non-malignant nature of the cells of Group A (clones 1, 5 and 7) was suggested by a low labeling index of [^{3}H]thymidine, characteristics of contact inhibition, lack of growth in soft agar and non-transplantability in mice. These results coincide with those of the study by Price and Tarin,[19] in which low incidence of transplantability in the mammary fat pads was observed in agarose colonies from spontaneous murine mammary tumors. The non-transplantability phenomenon was often found even after culturing for a long period. This might be due to the immunological variability of the viral antigen or to the elimination of transplantability after being cultured for such a long time.

The malignant nature of the cells of Group B (clone 6) was confirmed by their morphology, growth in soft agar and transplantability in mice. Cells of Group C (clones 3 and 4) showed intermediate characteristics, in which a peculiar morphology of dome formation, a non-colony forming capacity in soft agar, non-transplantability in the subcutaneous tissue and transplantability in the mammary fat pads and under the kidney capsule were observed. The transplantability in both tissues may be due to the fact that tumor growth is easier in a vessel-rich environment.

MMTV antigens are usually expressed only in mammary epithelial cells. In the cells of all six clonal lines, most MMTV antigens were detected as budding forms. Keratin filaments were detected in many cells of all of the lines after treatment with dibutyryl cyclic-AMP. These epithelial characteristics of cells were also confirmed by the presence of desmosome-like structures detected by electron microscope.

In the mouse strains such as C3H and BR6, MMTV is transmitted as a milk exogenous virus.[20] Rearrangements of endogenous MMTV provirus genomes or multiple insertion of exogenous MMTV proviruses frequently occur throughout the evolution of mammary cancer. The present study detected numerous exogenous MMTV proviruses in the clonal cells by Southern blot analysis with MMTV-*env* probe. The insertion pattern of these proviruses in Group C was quite different from those in Groups A and B. But the insertion pattern of Group A resembled that of Group B except for one extra band in Group B. These findings indicate that the cells of Groups A and B may have originated from a common ancestor *in vivo* or *in vitro*, although they were quite different in morphology and transplantability. There are two possible explanations. One is that the common ancestor was a non-malignant immortalized cell *in vivo* or *in vitro*, being transformed only in Group B for unknown reasons. Divergent subpopulations appear in the premalignant cell population, and some eventually emerge as malignant tumors. Another possibility is that the common ancestor of Groups A and B was malignant already *in vivo*, resulting in revertants only in Group A. On the other hand, Group C likely originated from different cells. The genetic plasticity of neoplasms might have led to the heterogeneity in the culture cells used in the present study.

Mammary tumorigenesis in the C3H and BR6 strains is characterized by a high frequency of MMTV proviral insertion into the common integration sites near the putative cellular oncogenes, *int*-1[8] and *int*-2[9]. Such a proviral activation of *int*-1[8] and *int*-2[9] induced the novel restriction fragments of these oncogenes in Southern blots analysis, and always leads to the expression of the oncogenes, although this expression occurs even without proviral activation. Therefore, we first examined proviral activation of *int*-1 or *int*-2 in the clonal cells. In the

Southern blot with *int*-1 or *int*-2, only one band (7.8 or 10.5 kb, respectively) was detected. This means that no proviral insertion occurred near *int* genes. Then we examined the expression of *int* genes in the clonal cells by Northern blot analysis. However, no significant band was detected in the blots (data not shown). These results suggest that mammary tumor transplantability is not primarily due to the two *int* oncogenes (*int*-1 and *int*-2), but might rather be due to other mammary oncogenes (*int*-3,[21] *int*-H[22] and *int*-41[23]) or unknown mammary oncogenes.

It is interesting that the cells that show contact inhibition of growth and no transplantability can be cocultured for a long time with other cells that show piling-up growth and transplantability. One possibility to explain the development of the immortalized non-malignant clonal cells is an epigenetic variation during *in vivo* tumor development or *in vitro* passage. Normal mammary cell lines may be important models to study mammary carcinogenesis. Only a few cell lines have been established from normal mammary gland.[6] Therefore, our cell lines of Group A which had many normal phenotypes, may be useful as experimental tools in the study of mammary carcinogenesis.

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