Insufficiency of Transformation by Simian Virus 40, Polyomavirus, EJ-*ras*, or v-*myc* Oncogenes for Conversion of Ethanolamine-Responsive Mammary Cells to Ethanolamine-Nonresponsive Cells

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Normal mammary epithelial cells (ethanolamine responsive) require ethanolamine to enable them to grow in defined culture medium because they cannot synthesize *de novo* a sufficient amount of phosphatidylethanolamine. Mammary tumor cells which retain properties of the normal tissue are also likely to be ethanolamine responsive, whereas dedifferentiated, highly tumorigenic mammary tumor cells are ethanolamine nonresponsive. The nonresponsive tumor cells are able to synthesize the necessary amount of phosphatidylethanolamine to sustain growth. Therefore, the progression of malignancy seems to convert ethanolamine-responsive mammary cells to ethanolamine-nonresponsive ones. In an attempt to prove the above assumption and to understand the mechanism responsible for the conversion during the progression of malignant transformation, mammary tumor cell line 64-24, which is typically ethanolamine responsive, was transfected with simian virus 40, polyomavirus, EJ-*ras*, or v-*myc* oncogenes, and the resulting transfectants were examined for their growth response to ethanolamine. Many of the transfectants exhibited typical transformed phenotypes; however, none of the transfectants converted to ethanolamine-nonresponsive cells. Some of the SV40 and polyomavirus transformants were able to grow in the absence of ethanolamine, although they grew better in the presence of ethanolamine, unlike typical ethanolamine-nonresponsive cells. These cells could grow in the absence of ethanolamine, even though their membrane phospholipid was phosphatidylethanolamine deficient. The present study indicates that the expression of any one of the four oncogenes tested, which allows the cells to exhibit transformed phenotypes in 64-24 cells, is not sufficient for the conversion of ethanolamine-responsive cells to nonresponsive cells.

Mammalian cells can be classified as ethanolamine (Etn) responsive or nonresponsive with regard to their proliferation in defined culture medium (for a review, see reference 19). Without supplemental Etn, Etn-responsive cells cannot synthesize a sufficient amount of phosphatidylethanolamine (PE); thus, the composition of their membrane phospholipid becomes PE deficient and develops a phosphatidylcholine (PC) excess. This is followed by a slowing down or cessation of growth (17, 20). The cessation of growth probably occurs because membrane-associated functions do not proceed properly in cells having an abnormal phospholipid composition (21). The Etn-responsive type includes epithelial cells of ectodermal and endodermal origins. Known cells of this type are normal rat, human, and mouse mammary epithelial cells (8, 13, 36); rat mammary carcinoma cell lines 64-24 and MTW9/PL (4, 12, 14); the human breast carcinoma cell line T47D (14); human epidermal keratinocytes and lung, bronchial, and prostate epithelial cells (26, 28, 33, 41); rat esophageal epithelial cells (1); rat pituitary tumor cells (36a); and mouse plasmacytoma, SP2/O, and all the hybridomas so far examined (15, 30). The Etn-nonresponsive type includes several mammary carcinoma cell lines of rats and humans (22-1, WRK-1, HBL100, MDA-MB231, and HS578T); rat hepatoma; rat, hamster, or mouse fibroblasts; and several rat neuronal and glial tumor cell lines (11, 15).

The most widely studied cells with respect to Etn requirement are mammary cells. According to the evidence so far accumulated, normal mammary epithelial cells and their tumor cell lines which tend to retain properties of the normal tissue (less progressed tumors) are Etn responsive, whereas

tumor cells which have lost many of the characteristics of normal mammary cells (progressed tumors) are Etn nonresponsive (10). Accordingly, we hypothesized that (i) normal epithelial cells require Etn to grow in culture, and (ii) a particular process(es) during the progression of malignancy renders the cells Etn nonresponsive, and therefore the loss of Etn responsiveness may be the one of pleiotropic manifestations of neoplastic transformation. If the above assumption is true, transformation of Etn-responsive mammary cells by a certain oncogene or oncogenes is expected to convert the cells from the Etn-responsive to the Etn-nonresponsive type.

In an effort to test the above assumption and to identify an oncogene(s) that confers Etn nonresponsiveness, we initiated transfection of rat mammary carcinoma cell line 64-24, which is typically hormone and Etn responsive (12, 18), by using four oncogenes, simian virus 40 (SV40), polyomavirus, EJ-*ras*, and v-*myc* oncogenes. The resulting transfectants were analyzed for their growth response to Etn, as well as for other characteristics. The results indicated two significant facts. (i) Some of the SV40 and polyomavirus transfectants became Etn nonresponsive, whereas all of the *ras* or *myc* transfectants remained Etn responsive. The membrane phospholipid composition of the SV40 transfectants, which became Etn nonresponsive, was, however, still PE deficient when the cells were cultured without Etn, indicating that SV40 T antigen and probably polyomavirus T antigens are capable of circumventing the PE deficiency of the cells, which is usually detrimental to the cells, by some other mechanism to support the growth. (ii) Many *ras* or *myc* transfectants manifested properties of characteristically transformed cells. However, none of them has converted to

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the Etn-nonresponsive type, indicating that the transformed phenotype is not sufficient for the conversion.

MATERIALS AND METHODS

Cells and cell culture. The present studies were carried out with a clonal rat mammary carcinoma cell line, 64-24 (18). 64-24 cells are of typical secretory epithelial cell morphology, and their growth is responsive to Etn and prolactin. They rarely form tumors in animals and hardly grow in soft agar, and the level of plasminogen activator is that of nontransformed cells (T. Kano-Sueoka and P. Hsieh, unpublished results; see below). The cells were normally maintained in high-glucose Dulbecco modified Eagle medium with penicillin (50 U/ml), streptomycin (50 µg/ml), 5% horse serum (Irvine Scientific, Santa Ana, Calif.), and 2.5% fetal calf serum (Irvine Scientific) at 37°C in a humidified atmosphere of air containing 10% CO₂ and were subcultured every 4 days. Anchorage-independent growth was tested by plating the cells in 0.35% agar containing the standard culture medium over the layer of 0.7% agar containing the medium. After 2 weeks of growth the colonies were stained with neutral red and counted.

Measurement of growth stimulation. Cells (5×10^4) were plated in duplicate or triplicate and grown in plastic tissue culture dishes (60 by 15 mm) either in serum-free medium described previously (16), except that 25 µg of bovine serum albumin per ml was used, or in Dulbecco modified Eagle medium containing 1.5% calf serum (Colorado Serum Co., Denver, Colo.) in the presence or absence of 5 µM Etn. The cells were released from plates with trypsin and counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.).

Plasmids. Five plasmids were used for transfection. Four of them carried a neomycin resistance marker (Neo^r). pSV3neo containing Neo^r and an entire early region of the SV40 genome (38) and pSV5neo containing Neo^r and an entire early region of the polyomavirus genome (38) were purchased from the American Type Culture Collection, Rockville, Md. pSV6.6neo containing EJ-ras, which is an allele of the human H-ras1 gene, was constructed by inserting a 6.6-kilobase BamHI fragment from pEJ6.6 (a gift from R. A. Weinberg, Massachusetts Institute of Technology, Cambridge [32]) into the BamHI site of pSV2neo (38) and confirmed by restriction mapping by C. Sackerson, University of Colorado, Boulder. The 6.6-kb fragment containing the EJ-ras gene includes its endogenous promoter. pSV7.3neo, which contains v-myc derived from MC29 avian myelocytomatosis virus, was similarly constructed from pSVv-myc (25, 42) (obtained from the American Type Culture Collection) and pSV2neo by C. Sackerson. The 7.3-kilobase insert from pSVv-myc has the MC29 long terminal repeat in the upstream region which controls the expression of v-myc gene. ptsA58 contains a temperature-sensitive SV40 T-antigen gene, tsA58 (39), and was obtained from L. Sompayrac, University of Colorado, Boulder.

Transfection and selection of transfectants. Plasmid DNA was prepared by the standard alkali-sodium dodecyl sulfate method (27). Essentially, the method described by Graham et al. (7), involving the calcium phosphate coprecipitation technique, was used to transfect 64-24 cells. To logarithmically growing cells, 25 µg of plasmid DNA suspended in 125 mM CaCl₂, 140 mM NaCl, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 0.75 mM Na₂HPO₄ (pH 7.2) was added, and the culture plates were incubated for 24 h. The cells were then washed, trypsinized, and plated with a series of dilutions. One day later, when the

cells were attached and started to proliferate again, 500 µg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml was added. Five days after the addition of G418, all untransfected cells were dead and the G418-resistant colonies were clearly observable. Individual G418-resistant clones were then isolated and propagated in the presence of 500 µg of G418 per ml. Once the clonal cell lines were established, the cells were maintained in the presence of 250 µg of G418 per ml. The frequency of transfection with regard to G418 resistance ranged between 3×10^{-6} and 7.5×10^{-4} . G418-resistant transformants with pSV2neo, which carries no oncogene, were also prepared as a control. Since ptsA58 does not carry the Neo^r marker, the cells were cotransfected with pSV2neo DNA as well as ptsA58.

Phospholipid analysis. The lipids were extracted from the cells in late log phase, and phospholipids were separated by two-dimensional thin-layer chromatography and quantitated as described before (20).

Expression of SV40 and polyomavirus T antigens and ras-p21. Expression of T antigens in the cells transfected with plasmid DNA containing SV40 or polyomavirus T antigens was examined by fluorescence microscopy. The primary antibodies used were PAB 419 monoclonal antibody against SV40 T antigen (a gift from E. Harlow, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) and polyclonal polyomavirus anti-T antibody (a gift from T. Benjamin, Harvard University, Boston, Mass.), and the secondary antibodies were fluorescein-conjugated, affinity-purified goat anti-mouse immunoglobulin G for SV40 T antigen and goat anti-rat immunoglobulin G for polyomavirus T antigen (Cooper Biomedical, West Chester, Pa.). The cells were grown on glass cover slips and fixed with acetone at -70°C for 10 min. The fixed cells were incubated with the primary antibodies for 30 min at 37°C in a humidified atmosphere, and after the cells had been washed twice, 100-fold-diluted secondary antibodies were used to stain the cells at 37°C for 30 min.

Monoclonal antibody against ras-p21 was obtained from Eastman Kodak Co., Rochester, N.Y. The cells grown on glass cover slips were fixed with acetone-methanol (1:1) at -20°C. The fixed cells were reacted with 50-fold-diluted primary antibody at room temperature for 30 min and then with 80-fold-diluted fluorescein-conjugated goat anti-mouse immunoglobulin G for 30 min.

Assay of plasminogen activator. The activities of plasminogen activator in the crude cell lysates were assayed by using a chromogenic substrate by the method developed by D. Abbott-Brown, Synergen, Boulder, Colo. (unpublished results). The cells were lysed with 0.5% Triton X-100 in sodium phosphate (pH 8.1). The reaction mixture (150 µl) contained 4 µg of human Glu-type plasminogen (American Diagnostica, Greenwich, Conn.), 40 µmol of Spectrozyme PL (American Diagnostica), and various amounts of the cell lysate in 0.05% Triton X-100 and 60 mM Tris buffer (pH 8.5). Microdilution plates were used to incubate the reaction mixtures at 37°C for 3 to 6 h, and the amount of the product was measured by its optical density at 405 nm with a Titertek Multiscan Spectrophotometer. Urokinase (Calbiochem-Behring, San Diego, Calif.) was used as the enzyme standard. The activities were expressed as units per milligram of protein. Each assay included the tumorigenic rat mammary carcinoma cell line 22-1, and the activities for other samples were normalized by taking the activity of 22-1 cells as 100.00.

FIG. 1. Phase-contrast micrographs of 64-24 cells transfected with SV40, polyomavirus, *v-myc*, and *H-ras* oncogenes. (A) 64-24; (B) Sv-3; (C) Sv-7; (D) Py-5; (E) Py-21; (F) My-5; (G) My-11; (H) Ra-2; (I) Ra-10. Bar, 100 μ m. The cells were grown in regular maintenance medium as described in Materials and Methods.

RESULTS

Transfections with the SV40 oncogene. pSV3neo DNA, which contains the SV40 oncogene, T-antigen gene, and Neo^r marker, was transfected into 64-24 cells as described in Materials and Methods. The frequency of transfection against Neo^r was 4×10^{-4} . Nine clonal lines of G418-resistant cells were established and analyzed for various properties. About half of these clonal lines looked very similar to the parental 64-24 cells, exhibiting a cobblestone-like smooth sheet of cells (Fig. 1A and B). Four of the lines,

on the other hand, had angular and irregular cell shapes and were less securely attached to the substratum (Fig. 1C). These cells tend to form irregular multilayer structures at confluency. Seven lines were tested for the presence of T antigen, and all of them were shown to be positive (representatives are shown in Fig. 2). In most cells the antigen was expressed in nuclei; however, in some cells only the cytoplasm contained the stain. There were variations in the extent of staining among the clonal lines as well as within the culture of a given line (Table 1; Fig. 2). Their ability to grow in soft agar was examined to see whether the expression of T antigen allows the cells to exhibit transformed phenotypes. These cloned lines hardly grew in soft agar under standard conditions, except one line, which grew at a low frequency (Table 1). Difficulty in growing rat cells transformed with SV40 has previously been reported (37). The level of plasminogen activator activity in these transfectants was therefore compared with that in untransfected 64-24 cells and its tumorigenic variant cell line, 22-1 cells (18). The intracellular and extracellular levels of plasminogen activator activity in 64-24 and 22-1 cells have previously been determined by D. Rifkin, New York University, New York, with ¹²⁵I-fibrin as the substrate. 22-1 cells have an 8- to 10-fold-higher activity than 64-24 cells do: the activity of 22-1 cells is that of typical malignant cells, and that of 64-24 cells is typical of nontumorigenic cells (31; D. Rifkin and T. Kano-Sueoka, unpublished results). In the present study, 64-24, 22-1, and four lines of the pSV3neo transfectants (all four are positive for SV40 T antigen) were used for the assay. 22-1 cells had about 10-fold-higher activity than nontransfected and transfected 64-24 cells did, and none of the transfectants contained significantly higher activities than the parental 64-24 cells did (Table 2).

The growth response of the nine clones to Etn was then examined to see whether the expression of T antigen conferred Etn nonresponsiveness on the cells. The response of individual lines to Etn was variable (Fig. 3A to E; Table 1). The growth property of four lines (Sv-2, Sv-3, Sv-4, and Sv-9) was very similar to that of 64-24 cells (Fig. 3A and E). Three (Sv-1, Sv-5, and Sv-8) were more dependent on Etn than 64-24 cells were (Fig. 3C), and, finally, two lines (Sv-6 and Sv-7) grew well without an Etn supplement (Fig. 3B and D). These two lines exhibit the morphology of transformed cells, and the levels of T antigen expressed, as examined by immunostaining, seemed to be higher than in the other cells. However, there are other lines, such as Sv-1 and Sv-9, which exhibit a transformed morphology or a line, such as Sv-5, which expresses T antigen at a high level, although their growth behavior is similar to that of nontransfected 64-24 cells. In addition, there is no distinction in the level of activity of plasminogen activator when comparing Etn-responsive and Etn-nonresponsive transfectants. Therefore, there seem to be no notable characteristics that distinguish transfectants which became Etn nonresponsive from those which remained Etn responsive.

Phospholipid compositions of the SV40 transfectants were examined to find whether the Sv-6 or Sv-7 lines, as a result of SV40 transformation, were able to grow in the absence of Etn because of their ability to synthesize sufficient PE whether the medium contains Etn or not. Accordingly, Sv-6, Sv-7, Sv-4, and untransfected 64-24 cells were grown in the presence or absence of 5 μ M Etn in Dulbecco modified Eagle medium containing 2% calf serum, and their phospholipids were analyzed as described in Materials and Methods. The results (Table 3) indicate that the manner in which the PE or PC content altered in all three transfected cell lines when

FIG. 2. Fluorescence staining of 64-24 cells transfected with SV40 oncogene with antibody against SV40 T antigen. (A) 64-24; (B) Sv-6; (C) Sv-1; (D) Sv-7. Bar, 100 μ m.

they were grown in the presence or absence of Etn was very similar to that of nontransfected 64-24 cells. Namely, these transfected cells were not able to maintain the normal level of PE or PC in the absence of supplemental Etn as typical Etn-nonresponsive cells are able to do. The results thus suggest that the Sv-6 and Sv-7 lines acquired the ability to grow in the absence of Etn, although their phospholipid composition is abnormal.

To ensure that the Etn nonresponsiveness acquired by Sv-6 or Sv-7 cells was due to the function of SV40 T antigen,

plasmid DNA containing a temperature-sensitive T-antigen gene, ptsA58, was transfected into 64-24 cells with pSV2neo, and the resulting G418-resistant transfectants (not cloned, two independently isolated but mixed populations of transfectants) were examined for their growth properties at 33°C (permissive temperature) and 39°C (nonpermissive temperature). Nontransfected 64-24 cells were clearly dependent on Etn for growth at both temperatures, and the dependence was more pronounced at 39°C (Table 4). Also, in the transfectants the cells behaved in a similar fashion as 64-24 cells at 39°C, whereas at 33°C both isolates of the transfectants grew rather well without Etn, although the addition of Etn shortened the generation time significantly (Table 4). Namely, at 33°C the generation times of transfected and nontransfected cells were very similar when they were

TABLE 1. Properties of 64-24 cells transfected with pSV3neo^a

Cell line	Morphology	Etn response ^b	Soft-agar growth (%) ^c	T antigen ^d
Sv-1	Transformed	++	<0.1	+
Sv-2	Parental	+	<0.1	NT ^e
Sv-3	Parental	+	<0.1	+
Sv-4	Parental	+	<0.1	+
Sv-5	Parental	++	<0.1	++
Sv-6	Transformed	—	1.4	++
Sv-7	Transformed	—	<0.1	++
Sv-8	Parental	++	<0.1	+
Sv-9	Transformed	+	<0.1	NT

^a Average frequency of transfection with respect to Neo^r was 3.5×10^{-4} .

^b Symbols: ++, more responsive than 64-24 cells; +, as responsive as 64-24 cells; —, nonresponsive.

^c The values were obtained by dividing the number of colonies formed in soft agar by the number of colonies obtained on a plastic surface.

^d Symbols: +, clearly positive; ++, greater than twice the + value.

^e NT, Not tested.

TABLE 2. Plasminogen activator activities of pSV3neo transfectants

Cell line	% Activity relative to 22-1 cells ^a
22-1	100.00
64-24	9.31 $\pm$ 1.76
Sv-1	10.29 $\pm$ 2.23
Sv-5	4.88 $\pm$ 0.32
Sv-6	5.94 $\pm$ 1.49
Sv-7	10.69 $\pm$ 1.70

^a 22-1 cells had an average activity of 372 U/ μ g of protein. Activities are expressed as means $\pm$ standard errors of seven separate assays. The activities were normalized by taking the activity of 22-1 cells as 100.00.

FIG. 3. Growth response to Etn of 64-24 cells transfected with SV40, polyomavirus, *v-myc*, and *EJ-ras* oncogenes. The cells were plated at a density of 5×10^4 cells per plate (60 by 10 mm) in serum-free medium or Dulbecco modified Eagle medium containing 1.5% calf serum with (—) or without (---) 5 μ M Etn. The cells were released from the plates with trypsin and counted in a Coulter counter. Each datum point represents the average of duplicate or triplicate samples. (A) 64-24; (B) Sv-7; (C) Sv-1; (D) Sv-6; (E) Sv-2; (F) Py-6; (G) Py-2; (H) Ra-29; (I) My-11; (J) 22-1.

grown in the presence of Etn, whereas in the absence of Etn the growth of 64-24 cells literally stopped, but transfected cells kept growing, with generation times less than twice the time of those grown with Etn. The above results strengthen the idea that acquisition of Etn independence by these transformants is due to the action of T antigen.

Transfection with the polyomavirus oncogene. The effect of DNA virus oncogene on 64-24 cells was further examined by transfecting the cells with plasmid DNA containing polyomavirus T-antigen gene, pSV5neo. A total of 22 Neo^r clonal lines were isolated, and 6 of them were examined for various characteristics as described above. Similarly to the pSV3neo transfectants, their morphologies ranged from those indistinguishable from that of parental 64-24 cells to those of typically transformed cells with angular and spiky shapes and less adherence to the plate (Table 5; Fig. 1D and E).

However, polyomavirus transfectants exhibited typical transformed morphology more frequently, and their transformed phenotype was more pronounced than that of SV40 transfectants (Fig. 1D). Our results agree with previous findings with rat fibroblasts that polyomavirus gives fully transformed phenotypes, whereas SV40 gives an intermediate level of transformation (34, 37). All of them so far tested were positive for the expression of polyomavirus T antigens (Fig. 4), and, unlike pSV3neo transfectants, all of the pSV5neo transfectants were able to grow in soft agar (Table 5). With regard to the growth response of these transfectants to Etn, one (Py-6) of six lines tested grew without supplemental Etn, but not as well as with Etn added as in the case of Sv-6 or Sv-7 lines (Fig. 3F). The majority of pSV5neo transfectants was more dependent on Etn than the 64-24 cells were (Fig. 3G). Notable characteristics associated with

TABLE 3. Phospholipid compositions of pSV3neo transfectants

Cell line	Etn	Mean amt (%) $\pm$ SE of:				
		SM ^a	PI ^b	PS ^c	PC	PE
64-24	—	2.5 $\pm$ 1.1	9.0 $\pm$ 2.5	10.7 $\pm$ 3.7	62.0 $\pm$ 8.2	15.8 $\pm$ 2.1
	+	2.7 $\pm$ 1.0	9.4 $\pm$ 1.2	13.0 $\pm$ 1.5	52.0 $\pm$ 2.3	22.7 $\pm$ 1.0
Sv-4	—	2.5 $\pm$ 1.0	7.7 $\pm$ 0.9	12.6 $\pm$ 1.4	63.9 $\pm$ 3.1	13.4 $\pm$ 0.8
	+	2.4 $\pm$ 0.8	9.1 $\pm$ 1.5	11.6 $\pm$ 1.2	52.4 $\pm$ 1.5	24.6 $\pm$ 4.2
Sv-6	—	1.6 $\pm$ 0.2	10.0 $\pm$ 0.5	10.5 $\pm$ 1.2	62.8 $\pm$ 2.6	15.3 $\pm$ 0.9
	+	1.7 $\pm$ 0.3	9.7 $\pm$ 1.5	11.2 $\pm$ 0.5	56.3 $\pm$ 1.2	21.1 $\pm$ 0.3
Sv-7	—	2.6 $\pm$ 1.1	9.1 $\pm$ 0.7	11.1 $\pm$ 0.8	60.2 $\pm$ 2.4	17.0 $\pm$ 1.0
	+	3.1 $\pm$ 0.9	6.6 $\pm$ 0.6	9.6 $\pm$ 0.8	52.0 $\pm$ 2.3	28.6 $\pm$ 1.0

^a SM, Sphingomyelin.

^b PI, Phosphatidylinositol.

^c PS, Phosphatidylserine.

TABLE 4. Growth response of ptsA58 transfectants to Etn at permissive and nonpermissive temperatures

Cell line	Temp (°C)	Generation time (hr) ^a	
		+ Etn	- Etn
64-24	33	12.0 ± 1.6	59.3 ± 13.2
	39	13.3 ± 0.9	NG ^b
ts-3A	33	13.3 ± 2.4	19.3 ± 1.3
	39	11.7 ± 1.3	NG
ts-3C	33	10.8 ± 1.3	19.3 ± 1.5
	39	11.5 ± 2.5	NG

^a Means ± standard errors of three or four separate experiments.^b NG, No growth.

their Etn-responsive growth was again not found as in the case of SV40 transfectants. The phospholipid composition was not analyzed for the Py-6 line, which was able to grow without Etn.

Transfections with EJ-*ras* and v-*myc* oncogenes. A total of 33 and 11 lines were isolated for pSV6.6*neo* (EJ-*ras*) and pSV7.3*neo* (v-*myc*) transfectants, respectively, and 6 representative lines of each were further examined. The morphology of most of the v-*myc* transfectants was very similar to that of the parental cells. Cells of one line, My-5, exhibited some transformed phenotypes when they were semiconfluent. However, the confluent culture of My-5 was virtually indistinguishable from that of 64-24 cells (Table 6; Fig. 1F and G). The growth in soft agar of these cells was generally negative except for one line, My-11, which grew at a low efficiency, and, finally, all lines needed Etn to grow in a similar fashion to 64-24 cells. Interestingly, My-11, which exhibits significant growth in soft agar, was more dependent on Etn than the parental line was (Fig. 3I).

The frequency of transfection for pSV7.3*neo*, which was selected for Neo^r, was unusually low (1.2×10^{-6} to 3×10^{-6}) compared with other plasmids. The reason may be that the majority of transfected cells contain multiple copies of *myc* DNA, and elevated levels of *myc* product are cytotoxic (43); therefore, only a few which do not contain excess plasmid DNA have survived. This may be the reason why literally all of the *myc* transfectants are similar to the parental cells.

In contrast to v-*myc* transfectants, transformed cells of literally all EJ-*ras* transfectants had distinct characteristic morphologies (Table 7). The cells had angular edges and

TABLE 5. Properties of 64-24 cells transfected with pSV5*neo*^a

Cell line	Morphology	Etn response ^b	Soft-agar growth (%) ^c	T antigen ^d
64-24	Parental	+	0.2	—
22-1	Transformed	—	21.1	—
Py-2	Parental	++	0.7	+
Py-3	Parental	++	4.7	+
Py-4	Transformed	++	2.8	+
Py-6	Transformed	—	11.9	++
Py-18	Transformed	++	10.1	++
Py-21	Transformed	+	8.2	++

^a Average frequency of transfection was 7.5×10^{-4} .^b Symbols: ++, more responsive than 64-24 cells; +, as responsive as 64-24 cells; —, nonresponsive.^c See Table 1, footnote c.^d See Table 1, footnote d. FIG. 4. Fluorescence staining of 64-24 cells transfected with polyomavirus oncogene. (A) 64-24; (B) Py-6; (C) Py-21. Bar, 100 μ m.

were less attached to the substratum (Fig. 1H and I), and two of six lines tested grew positively in soft agar (Table 7). All lines tested have clearly overproduced *ras*-p21 protein as tested by fluorescence microscopy (representatives are shown in Fig. 5). Further, the extent of *ras*-p21 production seems to be correlated with the efficiency of soft-agar colony formation (Fig. 5B and C). However, none of them became Etn nonresponsive or more dependent on Etn as a result of EJ-*ras* transfection.

DISCUSSION

Our previous observations regarding the Etn responsiveness of various mammary cells in culture led us to postulate

TABLE 6. Properties of 64-24 cells transfected with pSV7.3neo^a

Cell line	Morphology	Etn response ^b	Soft-agar growth (%)
My-4	Parental	+	<0.1
My-5	Transformed	+	<0.1
My-8	Parental	+	<0.1
My-9	Parental	+	<0.1
My-10	Parental	+	<0.1
My-11	Parental	++	1.1

^a Average frequency of transfection was 1.2×10^{-6} to 3×10^{-6} .

^b Symbols: ++, more responsive than 64-24 cells; +, as responsive as 64-24 cells.

that some step or steps during the progression of malignancy should alter the mammary cells from the Etn-responsive to the Etn-nonresponsive type. To test the above hypothesis, we have examined the effect of oncogenes on Etn-responsive mammary cells. If the results are positive, Etn nonresponsiveness could be used as a new parameter for tumor progression. Four oncogenes (two DNA virus and *myc* and *ras* oncogenes) were chosen for the present study.

Transfection with these oncogenes brought about some noticeable changes characteristic of transformed cells in the recipient mammary cells. In addition, 3 clonal isolates of SV40 and polyomavirus oncogene transfectants out of 15 so far tested were able to grow without supplemental Etn. The analysis of the growth response of the three transfectants seemingly converted to the Etn-nonresponsive type indicated that these cells were not completely independent of Etn as in the case of typically Etn-nonresponsive 22-1 cells (Fig. 3J), suggesting that the nature of Etn-nonresponsive growth of these transfectants may be different from that of typically Etn-nonresponsive tumor cells. Indeed, the analysis of phospholipids in these cells indicated that their ability to grow without Etn did not occur because they were able to synthesize sufficient PE *de novo* and hence maintain normal phospholipid composition. Fibroblasts such as mouse 3T3 or baby hamster kidney cells transformed with SV40 have been known to produce a protein growth factor(s) which stimulates the growth of the untransformed fibroblast cells (2, 3, 35). Accordingly, the ability of Sv-6, Sv-7, or Py-6 cells to grow in the absence of Etn in serum-free medium may occur because these transformants produce an autocrine growth factor which can stimulate the growth of the cells irrespective of their abnormal phospholipid composition. Preliminary results indicate that these cells indeed produce some kind of factor or factors which can stimulate the growth of parental 64-24 cells in the absence of Etn. The nature of the factor(s) is not known.

The hypothesis that acquisition of Etn-nonresponsive growth after transfection with SV40 or polyomavirus onco-

TABLE 7. Properties of 64-24 cells transfected with pSV6.6neo^a

Cell line	Morphology	Etn response ^b	Soft-agar growth (%)
Ra-2	Transformed	+	<0.1
Ra-3	Transformed	+	<0.1
Ra-10	Transformed	+	0.1
Ra-11	Parental	+	0.1
Ra-15	Transformed	+	5.0
Ra-29	Transformed	+	0.6

^a Average frequency of transfection was 6.5×10^{-4} .

^b +, As responsive as 64-24 cells.

FIG. 5. Fluorescence staining of 64-24 cells transfected with H-ras oncogene. (A) 64-24; (B) Ra-15; (C) Ra-29. Bar, 100 μ m.

genes is due to the expression of the T antigens was confirmed by using a mutant with a temperature-sensitive mutation of the SV40 T-antigen gene, *tsA58*. Growth patterns of the *tsA58* transfectants behaved according to our predictions; i.e., at the nonpermissive temperature the transfectants were dependent on Etn for growth, whereas at the permissive temperature they could grow in the absence of Etn. In this case, however, the cells were not completely independent or nearly independent of Etn as were Sv-6, Sv-7, and Py-6. This may be because mixed populations of transfectants selected for Neo^r were used for the growth analysis, and therefore the culture must consist of a mixture of Etn-responsive and Etn-nonresponsive types if the *ptsA58* transfection gave rise to a similar proportion of Etn-respon-

sive and Etn-nonresponsive types as the pSV3*neo* transfection did. An interesting observation regarding the behavior of 64-24 cells at different temperatures is that the cells are more sensitive to the Etn requirement at higher temperatures. Although the cells remain alive at 37°C in the absence of Etn, even if their growth stops, they cannot survive at 39°C without Etn. It may be that at 39°C a high PC content makes the cellular membrane too fluid to function properly or to maintain an intact cellular structure.

SV40 does not fully transform normal rodent cells (37). The present study indicates that the SV40 oncogene is not able to confer transformed phenotypes such as the ability to grow in soft agar or higher levels of plasminogen activator activity on 64-24 cells. In contrast to SV40, transfection with the polyomavirus T-antigen gene caused most of the cells to have typically transformed characteristics, as in the case of rat fibroblasts (34, 37). The effect of those DNA viral oncogenes on Etn responsiveness seems, however, very similar.

For the present study we have chosen two oncogenes, *myc* and *ras*, from a number of available oncogenes whose normal counterparts exist in the host cells, since these oncogenes are often activated in mammary tumor cells. The *myc* gene has often been found to be amplified in primary human breast carcinomas (6) or human breast carcinoma cell lines (23, 29). The *ras* genes are also often overexpressed in breast carcinoma tissues (9, 40). Particularly interesting is that the c-Ha-*ras* oncogene confers on the estrogen-responsive human breast carcinoma cell line, MCF-7, estrogen autonomy for growth both in culture and in animals (5, 22). Our results indicated that some *myc*-transfected cells and literally all of the *ras*-transfected cells manifested some characteristics of transformed cells. However, neither *ras* nor *myc* alone converted the Etn-responsive cells to Etn-nonresponsive cells. Introduction of the EJ-*ras* gene into 64-24 cells altered not only the morphology of the cells, but also their ability to grow in soft agar, suggesting that these cells have progressed at least a step further in the development of malignancy. Furthermore, the amount of *ras*-p21 present in *ras*-transfected cells seems to be correlated with the ability of the cells to grow in soft agar. However, this was not sufficient to convert the cells to Etn nonresponsiveness. *myc* transfection resulted in the least strong manifestation of transformed phenotypes in recipient cells. The *myc* gene product was not examined in these transfectants. However, since all of the Neo^r isolates for the transfectants from three other oncogene-containing plasmids expressed significant amounts of oncogene products, it can be reasonably assumed that the selected Neo^r cells for pSV7.3*neo* also integrated *myc* DNA. However, since it is highly possible that the reason for the low frequency of transfection with pSV7.3*neo* is the cytotoxicity of the *myc* gene product at elevated levels (43), surviving Neo^r isolates may not produce excess *myc* gene product. If the above is the case, the fact that cells converted to Etn nonresponsiveness were not found among *myc* transfectants may not be significant unless only a small amount of *myc* gene product is required for the conversion.

The present study suggests that oncogenes of DNA viruses are not capable of converting Etn-responsive mammary cells to Etn-nonresponsive cells and that conversion of the cells from Etn responsiveness to nonresponsiveness may require the activation of a cellular oncogene other than *myc* or *ras* or the activation of more than one oncogene. Rearrangement of chromosomes or the loss of the allele of one of the oncogenes in question may be necessary in addition to

the expression of oncogenes for converting cells from a responsive to a nonresponsive state. For example, the loss of a normal allele of the *ras* oncogene seems to be important for the progression of malignancy in breast cancer cells (24, 40). The examination of oncogenes other than the ones used in the present study and double transfections with various combinations of oncogenes are under way. The long-term culture of *ras* and *myc* transformants under conditions without Etn to select for Etn-nonresponsive cells is also in progress in order to examine the possibility that a secondary event after the introduction of *myc* or *ras* DNA is necessary for obtaining the Etn nonresponsiveness. This line of study should contribute to the understanding of the phenomenon of malignancy progression.

ACKNOWLEDGMENTS

We are grateful to Richard Frederick for able technical assistance. This work was supported by Public Health Service grant CA30545 from the National Institutes of Health and grant PCM8313161 from the National Science Foundation.

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