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Simian virus 40 large tumor antigen alone or two cooperating oncogenes convert REF52 cells to a state permissive for gene amplification

(12S E1A/*ras*/genetic instability/transformation)

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ABSTRACT **Gene amplification is characteristic of tumors and continuous cell lines but not of primary, normal, diploid, senescing cells. However, the rat cell line REF52, which resembles primary cells in requiring expression of cooperating oncogenes for transformation, is unusual among cell lines as it is not permissive for amplification. REF52 cells did not form colonies in *N*-(phosphonacetyl)-L-aspartate (PALA), a drug for which the only known mechanism of resistance is amplification of the carbamoylphosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD) gene. Colonies did form in a low concentration of methotrexate but did not contain amplified dihydrofolate reductase genes. Expression of two cooperating oncogenes in REF52 cells converted them to a state permissive for amplification. Cells expressing only the 12S E1A mRNA of adenovirus 5 did not give rise to PALA-resistant colonies, but expression of an activated *ras* gene together with E1A readily allowed the cells to form resistant colonies in which the CAD gene was amplified. Cells expressing E1A plus *ras* were fully transformed, but expression of simian virus 40 large tumor antigen alone converted REF52 cells to a state permissive for amplification without transforming them fully. The ability to manipulate gene amplification in REF52 cells by expression of oncogenes should contribute to an understanding of the nature of the permissive state.**

Gene amplification often provides the mechanism for overexpression of oncogenes in tumors and is also responsible for the ready overexpression of many genes whose products mediate drug resistance in cell culture (for a review, see ref. 1). However, primary cells in culture have not been observed to form drug-resistant colonies through gene amplification (2, 3). Since the vast majority of transformed and immortalized cells are permissive for amplification and since acquisition of these states requires the expression of oncogenes it seems likely that permissivity will also be affected by oncogenes.

Previous work has already suggested a genetic basis for gene amplification. Amplification rates differ among permissive cell lines, and variant lines with high rates, termed amplificator cells, have been selected (4). The amplificator phenotype can be transferred between different cells (5), and increased expression of c-*myc* stimulates amplification of the dihydrofolate reductase (DHFR) gene in established rat fibroblasts (6). Furthermore, there is evidence that amplification rates and tumorigenicity are correlated (7, 8), suggesting that expression of oncogenes other than c-*myc* may also influence amplification rates. It is also important to test the ability of oncogenes to affect permissivity for gene amplification, as distinct from their effect on the rate of this process.

The rat cell line REF52 resembles primary cells in requiring oncogene cooperativity for transformation (9–11). Like normal cells, REF52 cells are resistant to transformation by activated *ras* (9, 10). Most REF52 cells arrest upon transfection with *ras* and the few cells that continue to proliferate contain either a very high or a very low level of the activated *ras* gene product (9). Cells containing high levels of *ras* divide for about 20 generations and then die, whereas those with low levels continue to divide and can become morphologically transformed upon subsequent introduction of a cooperating oncogene (9). Several nuclear oncogenes such as E1A of adenovirus 5 (9) or large tumor antigen (T antigen) of simian virus 40 (SV40) (10) can cooperate with *ras* to transform REF52 cells. E1A alone does not transform either primary (12) or REF52 cells (9). A small fraction of REF52 cells expressing SV40 T antigen alone do become fully transformed, as defined by their high efficiency of growth in soft agar and ability to form tumors in nude mice (13). We generated populations of REF52 cells that express oncogenes by using retroviral vectors expressing the nuclear oncogenes E1A or T antigen alone or either of these together with a vector expressing *ras*. These populations differ markedly in their permissivity for gene amplification.

MATERIALS AND METHODS

Cell Lines. REF52 is an immortal line of postcrisis Fischer rat embryo cells (14). T24.10 and T24.12 are REF52 clones expressing an oncogene related to Harvey *ras* (Ha-*ras*) and derived from T24 human bladder carcinoma cells (9). These three cell lines were kindly provided by Robert Franza (Cold Spring Harbor). SVX-1, 12SE1A, TAG, E1Aras, and TAGras are populations of cells derived from REF52s infected with recombinant retroviruses expressing the relevant oncogenes. Cells were grown in Dulbecco's modification of Eagle's medium with 10% fetal calf serum (Bocknek Organic Materials, Rexdale, ON, Canada) at 37°C in an atmosphere of 10% CO_2 in air. A single batch of serum was used for all experiments.

Recombinant Retroviruses. The retroviruses ZipneoSV(X)1 (vector, ref. 15), ZipSV40 6 (SV40 early region, expressing T antigen alone; ref. 16), ZipE1A12S (adenovirus 5 early region, expressing the E1A 12S mRNA; ref. 17), and pBABE Hygro ras (encoding v-Ha-*ras*, ref. 18) were kindly supplied by Hartmut Land (Imperial Cancer Research Fund). All viruses conferred resistance to G418 except pBABE Hygro ras, which conferred resistance to hygromycin.

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Abbreviations: DHFR, dihydrofolate reductase; PALA, *N*-(phosphonacetyl)-L-aspartate; MTX, methotrexate; SV40, simian virus 40; T antigen, large tumor antigen; CAD, carbamoylphosphate synthetase/aspartate transcarbamoylase/dihydroorotase.

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Retrovirus Infection. REF52 cells were plated at 5×10^5 cells per 10-cm dish and infected the following day. Two milliliters of medium containing virus plus 8 μg of Polybrene per ml (Aldrich) was placed on the cells for 90 min at 37°C after which the cells were rinsed with 5 ml of medium. Fresh medium was added and, the following day, the cells were trypsinized and dispersed at 10^5 cells per 10-cm dish in the presence of 1 mg of G418 per ml (Geneticin, GIBCO) or 250 μg of hygromycin per ml (Calbiochem). The infection efficiency was ≈0.5% for ZipE1A12S and ≈0.2% for all the other viruses. Each drug-resistant population of cells contained several hundred individual transformants. E1A and T antigens were expressed, as demonstrated by immunoprecipitation (data not shown). In confirmation of these results, the populations of cells had characteristic morphology changes and were susceptible to transformation by *ras*.

Analysis of Frequencies and Rates of Gene Amplification. *N*-(Phosphonacetyl)-L-aspartate (PALA) was obtained from the Drug Synthesis and Chemistry Branch of the Division of Cancer Treatment, National Cancer Institute, and methotrexate (MTX) was from Sigma. The sensitivity of cells to each selective agent was defined as the concentration of drug that inhibited cell growth by 50% (IC_{50}) (19). Frequencies of drug resistance were determined at a density of 10^5 cells per 10-cm dish, using PALA concentrations equivalent to $3 \times IC_{50}$. A low cell density was necessary for good cell killing and to allow room for resistant colonies to grow. Cells were counted and plated immediately in medium containing selective agent. Plating efficiencies in medium containing dialyzed serum were determined at the same time. The frequencies of resistance have been corrected for plating efficiency. For analysis of amplification rates by the fluctuation method, cells were counted, plated in nonselective medium in microwells, and grown for 7 or 8 days, to ≈10^5 cells per well. The cells in a few wells were counted to determine the average of the number of cells selected (N). The cells in the remaining wells were dispersed into medium containing PALA and the contents of each well were transferred to a 10-cm dish. Rates were calculated by both methods of Luria and Delbrück (20), using the tables of Capizzi and Jameson (21), and were corrected for plating efficiency by the method of Stewart *et al.* (22). Since the results obtained by both methods were in good agreement, only the rates calculated by the method of the mean are reported. Drug-resistant colonies were cloned, grown in the same concentration of drug used for selection, and analyzed for gene copy number.

Analysis of Gene Copy Number. Southern analysis was carried out using established procedures (23). The carbamoylphosphate synthetase/aspartate transcarbamoylase/dihydroorotase (CAD) probe was the 6-kilobase (kb) *Hin*dIII fragment from the insert of pCAD142, which carries a partial cDNA for Syrian hamster CAD (24). The DHFR probe was a 3.2-kb repeat-free fragment of the Chinese hamster ovary DHFR gene (pMH8, from Lawrence Chasin, Columbia University, ref. 25). As a control probe, we used an insert from a plasmid containing the cDNA for the α_1 (pUCα1, ref. 26) or the β (rb19G, ref. 27) subunit of the rat Na,K-ATPase (from Robert Levenson, Yale University). Slot blots were carried out according to McIntyre and Stark (28). Density measurements were made with an LKB UltraScan XL instrument.

Growth in Soft Agar. Anchorage independence was measured as the ability of cells to grow in 0.35% low-melting-point agarose, layered over 0.6% agarose. Cells were plated at a density of 5×10^3 cells per 6-cm dish and colonies were counted after 3 weeks.

RESULTS

Gene Amplification Was Not Detected in REF52 Cells. Since amplification of the CAD gene is the only known mechanism of resistance to PALA (1, 29), we used this drug to test the ability of REF52 cells to amplify their DNA. No resistant colonies were observed (frequency, $<2.6 \times 10^{-7}$, Table 1) at a concentration of PALA ($3 \times IC_{50}$) minimal for selection of amplified CAD genes in cells known to be permissive for amplification (29, 30). In most cell lines, the frequencies of PALA resistance are about 10^{-5} (1).

Primary human (2, 3) and rat (2) cells, which have not been observed to amplify genes, do not form colonies in PALA but do in MTX. It is well known that resistance to MTX can be mediated through mechanisms other than amplification of the DHFR gene. REF52 cells do form drug-resistant colonies after 3 weeks in 50 nM MTX. The frequency was 1.8 or 5.6×10^{-5} in two experiments with ten 10-cm plates, each with 10^5 cells per plate. Ten such colonies were cloned, grown in the presence of MTX, and analyzed for DHFR gene copy number. Each clone had a number of DHFR genes equal to that of unselected REF52 cells (within 20%). Thus, none contained amplified DHFR genes. Stepwise selection to high concentrations of MTX has been used to select cells with many amplified DHFR genes (7). We tripled the concentration of MTX used to select resistant REF52 cells over a 1-month period, but the cells ceased to divide in the higher concentrations of drug. Similar results were obtained by Wright *et al.* (3), who failed to isolate colonies of normal cells in which resistance to MTX was due to amplification of the DHFR gene, and by Lücke-Huhle and Herrlich (31), who were not able to isolate colonies of differentiated F9 cells resistant to high levels of MTX. Thus, although REF52 cells can form stable colonies that do not appear to contain amplified DNA in drugs such as MTX and G418 (see below), they seem to be unable to form stable colonies that contain amplified DNA.

REF52 Cells Expressing E1A and Activated *ras* Are Permissive for Amplification. Since most cell lines are capable of gene amplification (for examples, see refs. 6, 7, and 29), we reasoned that expression of an immortalizing oncogene such as E1A might allow REF52 cells to amplify their DNA. We used retroviral vectors to introduce the oncogenes with high efficiency, thereby obtaining large pools of oncogene-expressing cells, which should give a more representative result than individual colonies. REF52 cells were infected with a retrovirus expressing the 12S mRNA from the E1A gene of adenovirus 5 (ZipE1A12S) and selected with G418. When a population of E1A-expressing cells was exposed to PALA, no resistant colonies were observed (Table 1).

We next tested the ability of *ras* to convert the E1A-expressing cells to a state permissive for amplification. pBABE Hygro ras, encoding the v-Ha-*ras* gene and hygromycin B phosphotransferase, was used to infect the G418-resistant 12SE1A population and the resulting cells were selected with hygromycin. This morphologically transformed population of cells (E1Aras) readily gave rise to PALA-resistant colonies (Table 1), and all such colonies that were tested contained amplified CAD genes (Fig. 1), as expected

Table 1. Frequency of PALA resistance in REF52 and derivative cells

Cells	PALA, μM	Total cells plated × 10^{-5}	Plating efficiency, %	No. of colonies
REF52	30	100	38	0
12SE1A	150	99	18	0
T24.10	36	88	41	0
T24.12	51	82	10	0
E1Aras	90	5	13	194

The selective concentrations of PALA used were $3 \times IC_{50}$, determined for each cell type. The reason for variation in IC_{50} for different cell types is not known.

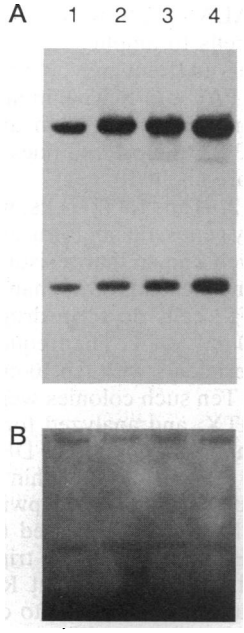

FIG. 1. Southern analysis of PALA-resistant E1Aras cells. Lane 1, DNA from E1Aras; lanes 2–4, DNA from three independent PALA-resistant clones derived from E1Aras. All of the DNA samples were digested with *Eco*RI. (*A*) Hybridization with a CAD probe. (*B*) Hybridization with a probe for Na,K-ATPase (loading control).

from previous work. The degree of amplification of CAD, 1.8- to 4.5-fold, was determined after each clone had been grown to $\approx 10^7$ cells in 90 μM PALA, the concentration of drug used for their selection.

The ability of E1Aras cells to amplify their DNA might be due either to expression of activated *ras* alone or to expression of *ras* and E1A together. Since REF52 cells expressing high levels of *ras* are not viable, we obtained two previously characterized clones of these cells (T24.10 and T24.12) that express low levels of activated T24 Ha-*ras* (9). Neither gave rise to PALA-resistant colonies (Table 1). Therefore, neither T24 Ha-*ras* alone nor 12SE1A alone can render REF52 cells permissive for amplification. Neither activated *ras* nor E1A alone is capable of transforming primary rat cells (11, 12) or REF52 cells (9), but together they transform such cells efficiently (9, 11, 12). Our data suggest that oncogene cooperativity is necessary to convert REF52 cells to a state permissive for amplification.

SV40 T Antigen Alone Converts REF52 Cells to an Amplification-Permissive State. SV40 T antigen transforms only a small subset of REF52 cells that express it (13, 16). Therefore, it was of interest to test whether T antigen alone would be capable of allowing REF52 cells to amplify their DNA or whether *ras* would be required in addition. We used ZipSV40 6, which expresses T antigen but not small tumor antigen (16). The resulting population of cells (TAG) was able to grow to a high population density but was not fully transformed, as measured by the ability of the cells to grow in soft agar (Table 2). TAG cells readily gave rise to PALA-resistant colonies (frequency, 3.5×10^{-4}) in which the CAD gene was amplified (data not shown). To measure the rate at which newly resistant cells were formed, a fluctuation analysis was performed (20). TAG cells formed PALA-resistant colonies at an appreciable rate, unlike parental REF52 or T24.10 cells, which gave no resistant colonies (Table 3).

Parental TAG cells do not grow in soft agar beyond two or three cell divisions (Table 2). Several independent PALA-resistant colonies derived from these cells were cloned and assayed for their ability to grow in soft agar. Two of six clones formed a few colonies visible to the naked eye (frequency, 0.4%; Table 2). Therefore, the ability to grow in soft agar does not correlate well with permissivity for amplification. Expression of activated *ras* (v-Ha-*ras*) in TAG cells generated a population of cells (TAGras) that did not have a higher rate of amplification than TAG cells (Table 3) but was morphologically transformed and able to grow well in soft agar (frequency, 4%). Therefore, the ability to grow in soft agar does not correlate with an increased rate of amplification in derivatives of REF52 cells.

Table 2. Anchorage independence of REF52 and derivative cell lines

Cell line	Visible colonies, %
REF52	0
SVX-1	0
12SE1A	0
E1Aras	5
TAG	0
TAG-PALA 1	0
TAG-PALA 2	0
TAG-PALA 3	0.4
TAG-PALA 4	0
TAG-PALA 5	0
TAG-PALA 8	0.4
TAGras	4

Cells (5×10^3) were plated in a 5-cm dish in agarose, and colonies visible to the naked eye were scored after 3 weeks. TAG-PALA clones resistant to 30 μM PALA were selected in a rate experiment (see text). The assays were repeated several times. In no case was a colony observed for cell lines that gave no colonies in the assay presented here.

DISCUSSION

Relationship Between Permissivity for Amplification and Oncogene Expression. Since gene amplification is a property of tumor cells and not normal cells, we have attempted to define genetically how a nonpermissive cell becomes permissive. Many immortal and transformed cell lines can become resistant to PALA, for which the only known mechanism is amplification of the CAD gene. The REF52 cell line is unusual in being nonpermissive for amplification, as shown by its inability to form colonies in PALA. This cell line also resembles primary cells in requiring cooperating oncogenes for transformation (9–11). When we introduced oncogenes into REF52 cells and measured the effect of their expression on amplification, we found that neither 12SE1A nor activated *ras* alone was sufficient to allow amplification of CAD genes, whereas coexpression of these two oncogenes did permit amplification. Even though *ras* plus 12SE1A did transform the cells, the permissive state for amplification does not

Table 3. Rates of PALA resistance, analyzed by fluctuation

Cell line	Events per cell per generation $\times 10^{-6}$
Experiment 1	
REF52	<1.7
T24.10	<2.2
TAG	21
Experiment 2	
TAG	43
TAGras	18

A total of 10^6 cells from each line was used in each experiment. The IC_{50} values for TAG and TAGras were 10 and 12 μM, respectively. The selective concentrations of PALA were 30 and 36 μM, respectively. PALA-resistant TAG and TAGras cells contained amplified CAD genes (data not shown).

require morphological transformation since expression of SV40 T antigen alone allowed REF52 cells to amplify their DNA without transformation, as measured by growth in soft agar. This result is not too surprising, as many untransformed cell lines are capable of amplification.

Our experiments do not address whether expression of T antigen is sufficient to convert primary cells to a state permissive for amplification. However, Lücke-Huhle *et al.* (32) did report that an SV40-immortalized human fibroblast line was not able to form stable colonies in high concentrations of MTX and that colonies resistant to low levels of MTX did not contain amplified DHFR genes. It appears that these cells are not permissive for amplification, although their ability to achieve resistance to PALA was not tested. Primary cells may require additional changes before they gain the ability to amplify DNA. Alternatively, the ability of SV40 T antigen to convert primary cells to a state permissive for amplification may be different for cells of rodents or primates or may be different for embryonic cells or fibroblasts.

Relationship Between Rates of Amplification, Transformation, and Tumorigenicity in Permissive Cells. Although we did not measure the tumorigenicity of the various derivatives of REF52 cells studied above, we did measure their ability to grow in soft agar, and similar derivatives of these cells have been assayed for tumorigenicity by others. REF52s, REF52s expressing 12SE1A, or REF52s expressing T24 Ha-*ras* do not grow in soft agar or form tumors when injected into nude mice (33). However, REF52 cells morphologically transformed by activated *ras* plus a cooperating oncogene do grow in soft agar and are tumorigenic (33). McClure *et al.* (13) showed that most clones of REF52 cells expressing SV40 T antigen do not grow well in soft agar or form tumors in nude mice. From these data, it is clear that anchorage independence correlates well with tumorigenicity in cells derived from REF52s. Our own study (Table 3) shows that the rate of amplification in TAGras cells is no greater than in TAG cells, even though only the former cells express both oncogenes, are morphologically transformed, and grow well in soft agar. Thus, neither permissivity for amplification nor an increased probability of amplification in permissive cells correlates well with the ability of derivatives of REF52 cells to grow in soft agar or to form tumors in nude mice (Table 4).

Possible correlations between high rates of amplification and increased tumorigenicity have been reported in several studies. Tlsty *et al.* (8) showed that the rate of amplification correlates with the ability of three rat cell lines to form tumors in syngeneic animals. Sager *et al.* (7) demonstrated that a Chinese hamster embryo fibroblast cell line (CHEF/16), which is diploid but tumorigenic, amplifies the DHFR gene at an enhanced rate compared to a closely related, diploid, nontumorigenic line (CHEF/18). Our data imply that heritable changes more subtle than acquisition of tumorigenicity govern permissivity for amplification and rates of amplification in permissive cells.

Table 4. Parameters of transformation in REF52 and derivative cell lines

Cell line	Growth in soft agar	Tumorigenicity*	Permissivity for amplification
REF52	−	−	−
SVX-1	−	−	−
12SE1A	−	−	−
T24.10	ND	−	−
E1Aras	+	+	+
TAG	−	−	+
TAGras	+	+	+

ND, not determined.
*Measured by others (13, 33).

Single oncogenes cause hyperplasia or dysplasia in reconstituted organs, whereas two cooperating oncogenes are tumorigenic (34). Double minute chromosomes, a hallmark of amplification in these tumors, are found in this situation and may contribute to tumor progression (34). T antigen fully transforms only a small proportion of the cells it immortalizes (13, 16), indicating that another event must occur before the cells become highly tumorigenic. It is possible that fully transformed cells arise as a result of amplification of a cellular oncogene in cells made permissive for this process by T antigen (16). These observations indicate that acquisition of a state permissive for amplification may be an early step in tumorigenesis and that this property may contribute subsequently to a more highly transformed phenotype.

Cellular Genes That May Be Involved in Permissivity for Amplification. We have shown that SV40 T antigen can convert a cell from a nonpermissive to a permissive state. T antigen is a multifunctional protein that affects several different properties of cells, including immortalization, transformation, patterns and rates of transcription, and control of DNA replication. Three functions of T antigen are needed for full transforming activity (35). Two of these can be provided by the protein product of 12SE1A mRNA (binding of Rb and the p300-associated function), whereas the third, binding of the tumor suppressor protein p53, is absent from E1A but present in the product of the cooperating adenovirus 5 oncogene E1B (35). REF52 cells express low levels of a p53 protein that is capable of binding to T antigen and are transformed by mutant p53 plus *ras* but not by *ras* alone (36). Both of these results indicate that the p53 gene is likely to be wild type in REF52 cells. Permissivity for amplification in REF52 cells may require that the tumor-suppressing, wild-type function of p53 be abolished since expression of T antigen, which binds to wild-type p53, allows amplification, whereas expression of E1A, which is not known to interact with p53, does not. It is certainly possible that more than one T antigen function is required to convert REF52 cells to a state permissive for amplification. It should be informative to test the ability of different mutants of T antigen that have lost individual functions for their ability to allow amplification in REF52 cells and then to correlate this property with the residual interactions between the mutant T antigens and cellular proteins. In this way, we may be able to identify cellular proteins involved in allowing cells to become permissive for amplification.

Denis *et al.* (6) have shown that overexpression of c-*myc* from a heterologous promoter increases the frequency of MTX resistance in established rat embryo fibroblasts, by amplification of DHFR and by other mechanisms. We did not test the ability of c-*myc* to convert REF52 cells to permissivity since exogenous *myc* cooperates poorly with *ras* to transform these cells (33) and the levels of endogenous c-*myc* mRNA are unaltered by expression of T antigen (33). However, it is still possible that some function of c-*myc* is altered in REF52 cells that express T antigen and that such a change contributes to the permissive state.

The primary events of amplification may happen very infrequently in nonpermissive cells. Many of the earliest events of CAD gene amplification in Syrian hamster cells involve recombination of the telomeric regions of sister chromatids to generate dicentric chromosomes, followed by bridge–breakage–fusion cycles (37). Counter *et al.* (38) have found that telomere shortening is associated with a greatly increased frequency of dicentric chromosomes in the development of immortal cell lines. Perhaps this is the crucial difference between the nonpermissive state of normal cells and the unusual REF52 cell line, on the one hand, and the vast majority of permissive cell lines, on the other.

Alternatively, a checkpoint in nonpermissive cells may prevent them from propagating when they contain highly

abnormal chromosomes containing amplified genes. Recently, it has become evident that the early events of CAD gene amplification involve very large segments of chromosomes, as shown by *in situ* hybridization to newly amplified genes in Syrian hamster cells (39). Rosenberger *et al.* (40) proposed that, in normal cells, a proofreading mechanism monitors chromosome segregation and replication and can inhibit cell growth when errors do occur. Permissive cells such as TAG and E1Aras may have lost this control.

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