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Suppression of tumorigenicity in hybrids of normal and oncogene-transformed CHEF cells

(Chinese hamster cells/*EJ* gene/genetics/protein p21/*ras* gene)

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ABSTRACT **Somatic cell hybridization experiments were carried out to determine whether normal cells have the ability to suppress the transforming effects of a defined oncogene. A nontransformed Chinese hamster embryo fibroblast cell line (CHEF/18-dm2) was used as the normal parent, and a CHEF/18 transfectant carrying the human mutant c-Ha-*ras* (*EJ*) oncogene was used as the tumorigenic parent. Selected hybrids (L318 cell lines) were assayed for the presence of *EJ* DNA, for the p21 product of the c-Ha-*ras* gene, and for various indices of cell transformation. These hybrids exhibited a fibroblastic morphology similar to the normal parent, although they contained the *EJ* gene and expressed its p21 protein product at levels comparable with the transformed parent. They had a reduced capacity for anchorage-independent growth (plating efficiency in methylcellulose of <0.3–13%, as compared with >90% for the transformed parent) and decreased tumor-forming ability in athymic mice. These findings show that normal CHEF/18 cells contain suppressor genes capable of inhibiting expression of the transformed phenotype, and tumor-forming ability, in the presence of an activated *EJ* oncogene.**

The cellular homologues of viral transforming genes, called proto-oncogenes, are expressed in many normal tissues and nontumorigenic cell lines (1), often in a cell-cycle-dependent manner (2), or in association with cell proliferation (3, 4). Since some proto-oncogenes induce the transformed phenotype when overexpressed (5, 6), their action must be well controlled in normal cells. Whether such an endogenous regulatory mechanism can also operate on activated oncogenes, such as the *EJ* (mutant c-Ha-*ras*) gene, is as yet unclear.

Somatic cell hybridization studies, involving the fusion of normal and transformed cells, suggest that normal cells *do* contain tumor suppressor mechanisms (for review, see ref. 7). For example, we have observed suppression in hybrids of normal and tumorigenic CHEF (Chinese hamster embryo fibroblast) cells (8) and in hybrids of normal and simian virus 40-transformed mouse cells (9). The suppression observed appears to have a genetic basis, since reemergence of tumorigenicity is associated with chromosome loss (10). In the case of the anchorage requirement, reversion to the nonsuppressed phenotype has been correlated with the loss of a specific chromosome (11).

We have initiated studies aimed at determining whether similar suppressor mechanisms are effective in the presence of a dominant-acting transforming gene—namely, the *EJ* gene. In the studies reported here, we fused normal CHEF/18 cells to an *EJ*-transformed CHEF/18 derivative. Our results show that transformation mediated by the activated *EJ* oncogene can be suppressed by normal cells.

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MATERIALS AND METHODS

Cell Culture. The CHEF/18 cell line and its CHEF/18-dm2 derivative (called dm2), which is resistant to thioguanine and ouabain, were described previously (12). The CHEF/18/EJ-L3 cell line (called L3) was derived from a focus recovered after transfection (13) of CHEF/18 cells with an *Eco*RI-linearized pSV2-*gpt*-*EJ* vector (14) containing the *EJ* insert (15). This line contains an average of one copy of the *EJ* gene per cell (unpublished data).

The growth medium for all cell lines consisted of α minimal essential medium supplemented with 10% fetal calf serum (Hyclone, Sterile Systems, Logan, UT), 2 mM glutamine, 100 units of penicillin/ml, and 100 μg of streptomycin/ml.

Cell Fusion and Hybrid Selection. Approximately 10^6 L3 cells were seeded on top of dm2 cells that had been inoculated at 10^6 cells per 25-cm^2 flask on the previous day. The monolayer was rinsed with phosphate-buffered saline after 9 hr and then treated for 35 sec with 48% (wt/wt) polyethylene glycol (Koch-Light Laboratories, Colnbrook Berks, England), which had been purified using a mixed bed resin (AG501-8XD, Bio-Rad Laboratories) as described by Yoakum (16). Treated cells were washed three times with serum-free medium, cultured overnight in growth medium, and plated (six dishes at 10^4 cells per 100-mm dish) into medium containing 0.1 mM hypoxanthine/0.45 μM aminopterin/20 μM thymidine/1 mM ouabain. (Hypoxanthine, aminopterin, thymidine, and ouabain were purchased from Sigma.) Forty-eight hybrid colonies (L318 cell lines) were isolated 2 wk later, the overall frequency of hybrid formation being 1.25×10^{-3}. Fifteen of these cell lines grew poorly or appeared unhealthy. The studies reported here were carried out using hybrids chosen from among the remaining lines, selection being based on nontransformed or fibroblastic morphology, the ability to grow in medium containing mycophenolic acid at 25 μg/ml (Eli Lilly), and tetraploid DNA content as determined by flow microfluorimetry.

Assay for *EJ* Sequences in Cell DNA. Genomic DNA was extracted as described (17, 18). Approximately 10 μg of DNA was digested sequentially with *Bam*HI (New England Biolabs) and *Sst* I (Bethesda Research Laboratories). Agarose gel electrophoresis, transfer to nitrocellulose filters, hybridization to the ^{32}P-labeled *EJ* probe [a 6.6-kilobase (kb) *Bam*HI *EJ*-specific fragment], and autoradiography were carried out using standard methods (18).

Assay of p21 Encoded by *EJ*. Labeling of cells with [^{35}S]methionine and immunoprecipitation of p21 were carried out using minor modifications of published procedures

Abbreviations: CHEF, Chinese hamster embryo fibroblast; *EJ* gene, c-Ha-*ras* transforming gene from the EJ human bladder carcinoma cell line; c-Ha-*ras*, cellular homologue of the transforming gene of Harvey murine sarcoma virus; kb, kilobase(s).

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(17, 19). Immunoprecipitation was carried out with 1.5×10^7 cpm of trichloroacetic acid-precipitable material using the rat monoclonal antibody preparation 238 (20), provided by Mark Furth. Immunoprecipitates were electrophoresed in a 12.5% polyacrylamide slab gel. Normal rat serum (Cappel Laboratories, West Chester, PA) or another rat monoclonal antibody (m169.16.11 HL2), from Timothy Springer, served as controls.

Assay of Cell Growth Properties. The growth rates and saturation (maximal) densities of parental and hybrid cell lines were determined by counting cells daily (Coulter Counter), after inoculating 2×10^4 cells onto 35-mm tissue culture dishes. Cells were fed twice weekly.

Plating efficiencies under anchorage-independent conditions were estimated as described (8, 11), by culturing 10^2–10^5 cells in medium containing 1.3% methylcellulose, using 60-mm dishes coated with 0.6% agar. Cells were fed weekly, and macroscopic colonies were scored at 4.5 wk. Plating efficiencies on plastic were determined by inoculating 100 cells onto 60-mm dishes, which were stained and scored at 2 wk.

Assay of Tumor-Forming Ability in Athymic Mice. The tumorigenicity of various hybrids was compared with that of parental cells by using a modification of the coinjection method developed in our laboratory (10). Test cells (10^6) were injected together with CHEF/18 cells or primary mouse embryo cells (10^7), which had previously been treated for 2 hr with mitomycin C at 4 μg/ml (Sigma). Cells were injected subcutaneously (in 0.2 ml of growth medium) into the flanks of athymic BALB/c mice bred in our laboratory. Tumors attaining an average diameter [(length + width)/2] of >0.6 cm were scored as positive. Tumors that did not reach this diameter within 8 wk were classified as slowly developing tumors. Mitomycin C-treated carrier cells did not form tumors when injected alone. With the parental cells, essentially identical results were obtained when tumorigenicity was tested by this coinjection procedure or by direct injection of larger inocula ($4–10 \times 10^6$) of test cells (10).

RESULTS

Morphology of L318 Cell Hybrids. Hybrid cell lines grown in monolayer exhibited some morphological variability. The CHEF/18-dm2 parent has a typical fibroblastic morphology, and a parallel, contact-inhibited, or "wheatsheaf" cell alignment, as shown in Fig. 1*A*. The *EJ*-transformed parent, in contrast, displays a shortened or rounded cell shape (Fig. 1*B*) and grows in a disorganized fashion to high cell density. About 27% of the hybrids appeared fibroblastic and morphologically most similar to dm2 (Fig. 1 *C* and *D*). Approximately another 45% were intermediate in morphology, generally fibroblastic but with one or more properties not typical of the normal parent. For example, some hybrids were slightly shortened or refractile. Others, although fibroblastic in character, formed a densely packed monolayer or exhibited cell overlap, small whorls, or raised areas.

Hybrid lines that appeared morphologically normal or near normal in monolayer culture were examined after growth as individual colonies. Colonies of dm2 cells (Fig. 2*A*) exhibit an ordered end-to-end cell alignment, with little overgrowth. In colonies of L3 cells (Fig. 2*B*), on the other hand, cells are arranged in a disorganized random fashion. These cells tend to overgrow, resulting in the appearance of very densely packed colonies. Colonies of hybrid cells, although in some cases quite densely packed, exhibited an

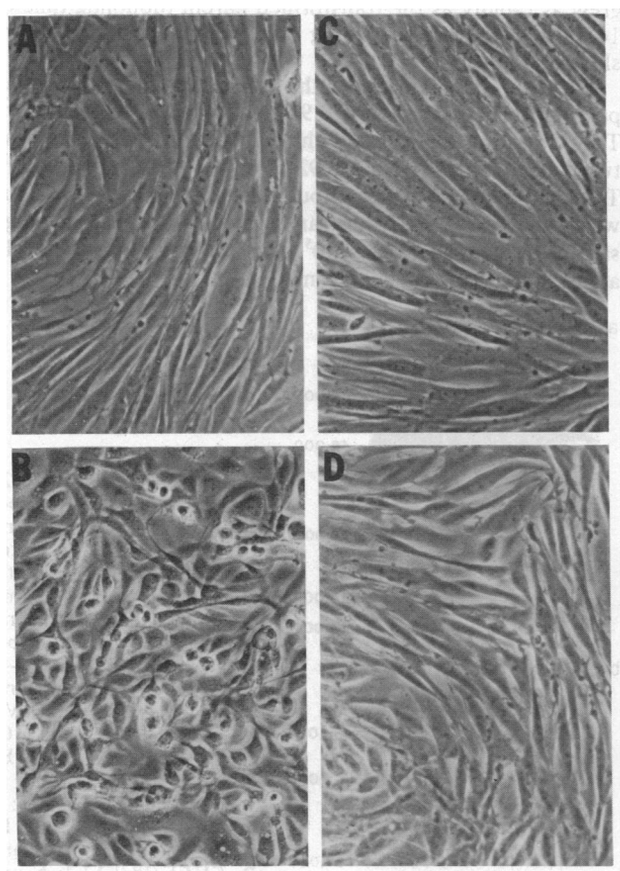

FIG. 1. Monolayer morphology of L318 hybrids between CHEF/18-dm2 and CHEF/18/EJ-L3. *A*, CHEF/18-dm2; *B*, CHEF/18/EJ-L3; *C*, L318-17; *D*, L318-5.

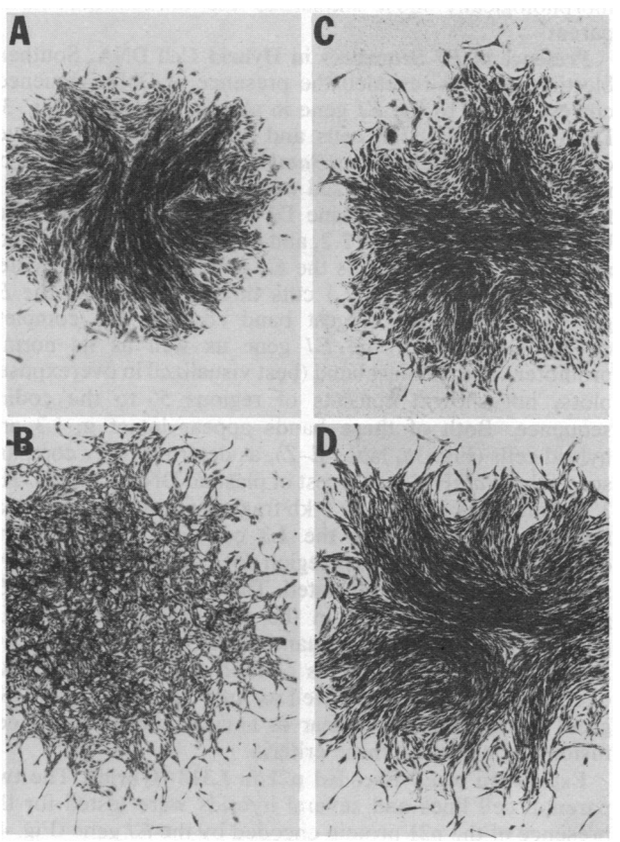

FIG. 2. Colony morphology of L318 hybrids between CHEF/18-dm2 and CHEF/18/EJ-L3. *A*, CHEF/18-dm2; *B*, CHEF/18/EJ-L3; *C*, L318-26; *D*, L318-13.

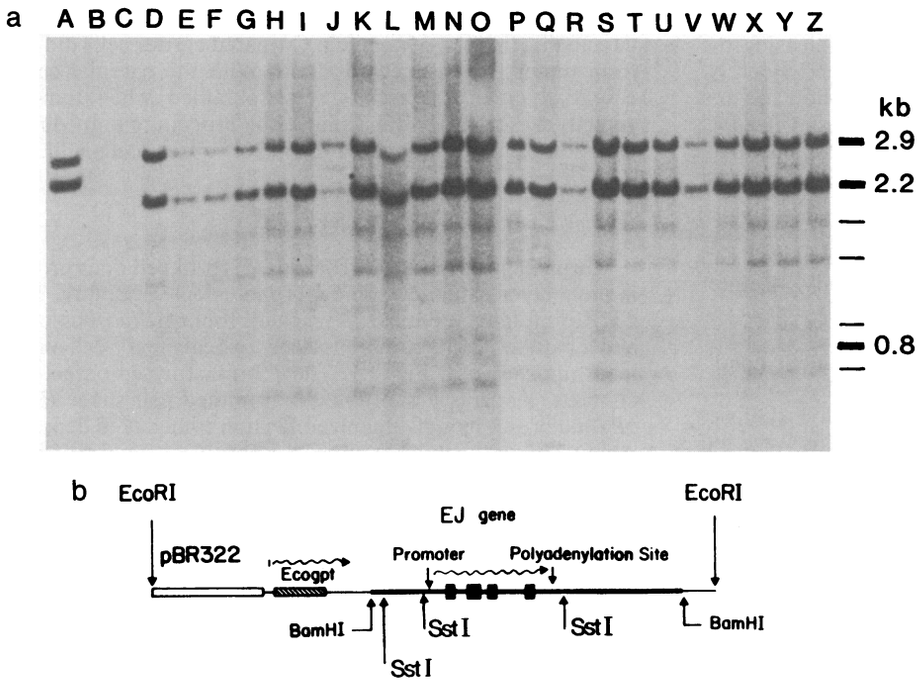

FIG. 3. Presence of *EJ* DNA in L318 hybrids between CHEF/18-dm2 and CHEF/18/EJ-L3. Genomic DNA from parental and hybrid cell lines was digested with *Bam*HI and *Sst* I, electrophoresed on a 1.0% agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated ^{32}P-labeled 6.6-kb *Bam*HI fragment of pEJ. (*a*) Lanes: A, CHEF/18 DNA with 10 pg of pSV2-*gpt-EJ* plasmid; B, CHEF/18; C, CHEF/18-dm2; D, CHEF/18/EJ-L3; E, L318-1; F, L318-4; G, L318-5; H, L318-6; I, L318-11; J, L318-12; K, L318-13; L, L318-14; M, L318-17; N, L318-20; O, L318-22; P, L318-26; Q, L318-31; R, L318-32; S, L318-33; T, L318-34; U, L318-36; V, L318-37; W, L318-45; X, L318-46; Y, L318-47; Z, L318-48. (*b*) Restriction and functional map of the pSV2-*gpt-EJ* vector used for transfection of CHEF/18/EJ-L3.

ordered cell alignment similar to that seen with the normal parent (Fig. 2 *C* and *D*). A small percentage of transformed colonies was observed, but this was <5 (at passage 2) for most hybrids. No transformed colonies were seen with the dm2 cell line, while 83% of L3 colonies were fully transformed and an additional 11% of colonies were semitransformed in appearance. Thus, these hybrid cell lines were morphologically most similar to the normal fibroblastic parent.

Presence of *EJ* Sequences in Hybrid Cell DNA. Southern blotting analysis revealed the presence of DNA sequences corresponding to the *EJ* gene in all hybrids tested (Fig. 3). DNA from CHEF/18 cells and all CHEF/18 derivatives contained four endogenous *Bam*HI/*Sst* I bands (indicated by narrow lines) that hybridized weakly to the *EJ* probe. DNA from L3 cells (Fig. 3*a*, lane D) had three additional dark bands (heavy lines), 2.9, 2.2, and 0.8 kb in size. These bands arise because *Bam*HI cuts the *EJ* gene from the transfected plasmid and because *Sst* I cuts three times within the *EJ* gene (Fig. 3*b*). The largest band contains the complete coding sequence of the *EJ* gene as well as its normal promoter. The smallest band (best visualized in overexposed blots, not shown) consists of regions 5′ to the coding sequence. Both of these bands appear intact in L3 and hybrid cells (Fig. 3*a*, lanes E–Z), as determined by comparison with a *Bam*HI/*Sst* I digest of plasmid DNA (lane A). The 3′ region of the *EJ* gene (2.5-kb fragment in the plasmid) was shortened to 2.2 kb in the L3 cell line and its hybrid derivatives. However, this region does not contain structural sequences and is not translated. The copy number of the *EJ* gene in the hybrids was in the same range as in the L3 parent, as determined by quantitative Southern blot analysis. Thus, the L318 hybrids contain the entire coding sequence of the *EJ* gene as well as its 5′ flanking region. No gross rearrangements appear to have occurred during cell fusion, as judged by these criteria.

Expression of *EJ*-Encoded p21 in L318 Hybrids. The two parental cell lines and several hybrids were tested for the presence of the p21 protein encoded by the *EJ* gene (Fig. 4). As shown, the *EJ*-encoded p21 product was readily detectable in L3 cells and in the hybrid cell lines at comparable levels. These results indicate that the *EJ* oncogene donated by the transformed parent is transcriptionally and translationally active in the hybrid cell lines.

Growth and Anchorage Dependence of L318 Hybrids. The growth properties of six representative hybrids are listed in Table 1. As indicated, the *EJ*-transformed parent proliferated more rapidly than the dm2 parent. Four of the hybrids grew as rapidly as the transformed parent (doubling time of 14–16 hr), and the remaining two grew only slightly more slowly.

The saturation densities of the normal and the transformed parents were, respectively, 0.92 and 2.1 × 10^5 cells per cm^2. The saturation densities of the hybrids fell between these two values, ranging from 0.92 to 1.4 × 10^5 cells per cm^2. Thus, although the average doubling time of hybrids (16.5 hr) was similar to that of the transformed parent, the average saturation density (1.2 × 10^5 cells per cm^2) more closely approximated that of the normal parent.

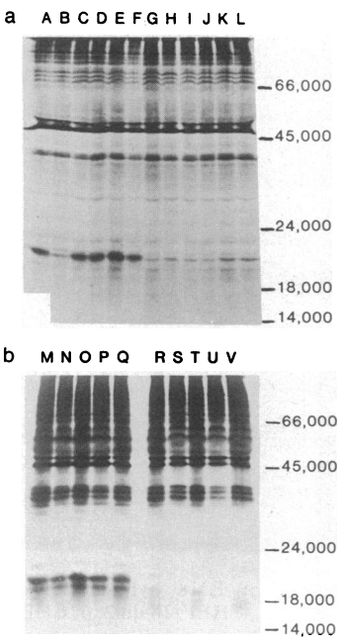

FIG. 4. Presence of p21 protein in L318 hybrids between CHEF/18-dm2 and CHEF/18/EJ-L3. Lysates from ^{35}S-labeled parental and hybrid cells were immunoprecipitated with a rat monoclonal antibody specific for Ha-*ras* (lanes A–F and M–Q) or with normal rat serum (lanes G–L and R–V). (*a*) Lanes: A and G, CHEF/18/EJ-L3; B and H, CHEF/18-dm2; C and I, L318-5; D and J, L318-13; E and K, L318-22; F and L, L318-37. (*b*) Lanes: M and R, CHEF/18/EJ-L3; N and S, L318-26; O and T, L318-32; P and U, L318-34; Q and V, L318-45.

Table 1. Growth characteristics of L318 hybrids between CHEF/18-dm2 and CHEF/18/EJ-L3 cells

Cell line	Doubling time, hr	Saturation density, cells × 10^{-5}/cm^2
CHEF/18-dm2	22	0.92
CHEF/18/EJ-L3	16	2.1
L318-5	15	0.92
L318-13	14	0.95
L318-22	18	1.4
L318-32	16	1.4
L318-37	20	1.1
L318-45	16	1.2

Cells were plated at 2 × 10^4/35-mm dish and fed twice weekly. Doubling time and saturation density were determined from cell counts taken during logarithmic and stationary phases, respectively.

The capacity of the hybrids for anchorage-independent growth was also intermediate between that of the two parents, as shown in Table 2. Although the normal parent plated very poorly in methylcellulose, and the transformed parent plated very well (methylcellulose plating efficiency, >90%), the plating efficiencies of the hybrids ranged from <0.3% to 13%. L3 cells grown in methylcellulose for 4 wk formed readily visible colonies that were predominantly intermediate or large in size. Numerous microscopic colonies, which may represent satellites, were also observed. Colonies formed by the hybrid cell lines were variable in size, but, overall, were smaller than those formed by L3 (Table 2). These data show that L318 cell hybrids have a reduced capacity for anchorage-independent growth, both colony number and colony size being decreased below the value obtained with the transformed parent.

Tumor-Forming Ability of L318 Hybrids. The dm2 parent was nontumorigenic in nude mice, while the L3 parent consistently formed tumors (Table 3). Tumor latency was 2–5 wk, death of the host generally occurring at 9–15 wk.

The hybrid cell lines were markedly less tumorigenic than the L3 parent. One hybrid (L318-45) did not form progressively growing tumors at any of seven sites assayed. With three additional hybrids (L318-1, L318-11, and L318-37), only a single tumor was formed out of five or six sites tested. Some hybrids formed slowly developing tumors: For example, L318-22 produced only two tumors out of seven sites tested, and these did not reach a size of >0.6 cm for 13–14 wk. Most of the remaining hybrids were negative at 30–70% of the sites tested and developed tumors of long latency at some of the positive sites.

DISCUSSION

We have tested the ability of normal CHEF/18 cells to suppress transformation in an *EJ*-transfected CHEF/18 derivative. Our results show partial to complete inhibition of transformed morphology, anchorage independence, and tumor-forming ability in normal–tumor cell hybrids, consistent with the hypothesis that normal cells contain genes capable of suppressing the activated *EJ* oncogene. Since these effects occur in the presence of continued p21 expression, the suppressor gene products do not act by interfering with oncogene transcription or translation. Instead, they may interact with, modulate, or compete with the oncogene product or may act at a different point within a chain of events leading to transformation and tumorigenicity.

Chromosome studies (unpublished data) of the parental cells and hybrids revealed a translocation [t(3p;8q)] present in the L3 cells and in all of the hybrid lines. As yet no additional rearrangements have been found, nor are there nonrandom losses of particular chromosomes in the near-tetraploid hybrid populations. These findings are consistent with other chromosome studies of suppressed hybrids (7), showing that suppression is a property of the total hybrid genome.

The finding of suppression in oncogene-transformed CHEF/18 cells is consistent with data, from this laboratory and others, showing that fusion of normal and tumorigenic cells results in decreased tumorigenicity (7). This phenomenon has been observed using tumor cells that are karyotypically normal, such as CHEF/16 (8), as well as with highly aneuploid cells (21). Suppression has been reported in simian virus 40- and avian sarcoma virus-transformed cells (9, 22) and in the HT-1080 cell line, which contains an activated N-*ras* gene (23). An analogous loss of malignancy is seen in revertants of Kirsten murine sarcoma virus-transformed cells (24, 25), and these cells can suppress transformation induced by related oncogenic viruses (24). In this case, suppression occurs in the presence of continued expression of the viral transforming genes, paralleling the situation seen with hybrids of CHEF cell transfectants. Similarly, normal human fibroblasts that have been transfected with the *EJ* gene express high levels of p21, although they are not transformed in phenotype nor tumorigenic in nude mice (17).

Table 2. Anchorage requirement of L318 hybrids between CHEF/18-dm2 and CHEF/18/EJ-L3 cells

Cell line	Plating efficiency, %		Ratio (methylcellulose/ plastic)	Colony size
	Plastic	Methylcellulose		
CHEF/18-dm2	30	0.04	0.001	Very small
CHEF/18/EJ-L3	52	>90.0	>1.0	Intermediate to large
L318-1	18	4.6	0.25	Very small
L318-5	38	8.6	0.22	Small
L318-11	28	10.9	0.38	Small to intermediate
L318-13	27	4.8	0.18	Very small
L318-17	41	8.8	0.21	Small to intermediate (a few large colonies)
L318-22	32	8.2	0.26	Very small
L318-26	34	10.0	0.29	Small to intermediate
L318-32	26	6.9	0.26	Small
L318-34	28	13.0	0.46	Small to intermediate (a few large colonies)
L318-37	31	<0.3	<0.01	Very small
L318-45	19	10.0	0.53	Intermediate

Cells were plated at 100/60-mm plastic tissue culture dish or at 10^2 to 10^5/60-mm agar-coated dish in methylcellulose medium. Plating efficiency on plastic was determined after 2 wk. Plating efficiency and colony size in methylcellulose were determined at 4.5 wk.

Table 3. Tumor-forming ability of L318 hybrids between CHEF/18-dm2 and CHEF/18/EJ-L3 cells

Cell line	Tumors/sites tested
CHEF/18-dm2	0/2
CHEF/18/EJ-L3	9/9
L318-1	1/5
L318-5	0/1
L318-11	1/6
L318-13	3/6
L318-17	2/3
L318-22	2/7
L318-26	2/6
L318-32	2/7
L318-34	2/3
L318-37	1/5
L318-45	0/7

Approximately 10^6 test cells were coinjected with 10^7 CHEF/18 cells (or primary mouse embryo fibroblasts) that had been inactivated with mitomycin C. With L318-13, L318-17, L318-26, and L318-32, one of the positive sites indicated was slow-developing, and both positive sites were slow-developing with L318-22. No slow-developing tumors were seen with CHEF/18/EJ-L3. One tumor regressed with hybrids L318-11, L318-32, and L318-34, and two tumors regressed with CHEF/18/EJ-L3. With hybrids L318-1, L318-11, L318-22, L318-26, L318-32, and L318-45, lumps of ≤ 0.6 cm appeared transiently at some additional injection sites.

Suppression of *EJ*-mediated transformation, although not unexpected on the basis of previous somatic cell hybridization studies, is nevertheless surprising in view of the fact that this gene appears dominant-acting on transfer into immortalized cells (13, 26, 27). Can the transforming effect of this gene in transfection experiments be reconciled with its suppressibility in cell hybrids?

The simplest hypothesis to invoke is that of competition between normal and mutant forms of the Ha-*ras* gene. L3 cells contain, on an average, one copy of the mutant *EJ* gene, which on this hypothesis is dominant over the two normal Ha-*ras* genes in L3, but not dominant over the four normal copies in the hybrids. Against this view is the evidence that normal Ha-*ras* driven by a strong promotor has the same transforming effect as the mutant gene (5). The hypothesis we favor is that two classes of genes are involved: *oncogenes* that facilitate and *anti-oncogenes* that suppress the tumorigenic transformation. Thus, suppressor genes (i.e., anti-oncogenes) may have been lost or inactivated in the oncogene-transformed parent. This may have occurred prior to transfection (predisposing to focus formation), during oncogene integration, or in conjunction with subsequent genomic changes. In *EJ*-transfected CHEF/18 cells, for example, chromosome aberrations are seen frequently in focus-derived cells and invariably after tumor formation (unpublished data). Thus, we postulate that suppressor gene action has already been lost in L3 cells and subsequently is regained in hybrids by fusion with normal cells.

In conclusion, these data provide evidence for the existence of anti-oncogenes, whose gene products, when identified, may provide new agents for cancer therapy. In most somatic cell hybridization experiments carried out in the past, it was not possible to control, or even identify, the specific genetic loci active in the tumorigenic parent used. This resulted in the difficulty of attempting to study suppressor genes, active against transforming genes that were themselves unidentified. The use of a system, such as the one described here, in which defined sequences are implicated in tumor formation should greatly facilitate the isolation and characterization of suppressor genes.

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