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Genetics

Resistance of human cells to tumorigenesis induced by cloned transforming genes

(simian virus 40/*EJ* gene/oncogene/transfection)

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ABSTRACT The transformation of human cells was examined by transfection of cloned oncogenic DNAs derived from the tumor virus simian virus 40 and from the human bladder carcinoma cell line EJ into diploid fibroblasts derived from foreskin (FS-2 cells). The simian virus 40 DNA was found to induce a morphologically transformed phenotype, leading to easily detectable focus formation. Tumor antigen was produced, but the transformed cells were not tumorigenic in the *nude* mouse. The *EJ* gene, a mutant form of the cellular c-Ha-*ras* gene, actively transforms NIH/3T3 mouse cells and CHEF/18 hamster cells but is inactive in FS-2 cells. Morphological transformation, focus formation, and tumorigenicity in *nude* mice were not induced when *EJ* DNA was transfected into FS-2 cells by using the selectable vector pSVgptEJ. The intactness of the transfected EJ DNA was established by restriction fragment analysis. This result raises the question of what role, if any, the mutated gene derived from the *EJ* cells played in the origin of the EJ bladder carcinoma.

Modern cancer research has been caught for some years on the horns of a dilemma: the concept of neoplastic transformation in a single step based largely on the studies of tumor viruses in animal systems and the vast evidence, both clinical and experimental, of neoplasia as a multistage process. A synthesis of these opposing views has begun as a consequence of the availability of a new set of analytical reagents called oncogenes, which are cloned fragments of DNA with transforming activity.

Oncogenes have been recognized by two kinds of experiments: identification by mutational analysis of transforming sequences in the genomes of avian and rodent retroviruses and identification of transforming sequences (in some instances related to retroviral transforming genes) in DNAs of tumor origin by their ability to induce oncogenic transformation of NIH/3T3 mouse cells (1). These experiments have been interpreted as support for the single-step origin of tumorigenicity, but the recipients being tested were either preneoplastic cells (e.g., NIH/3T3) that had already undergone unspecified genomic changes or animals infected with multifunctional viruses (i.e., containing long terminal repeats as well as transforming genes). Recently, transfection experiments using early passage rodent cells instead of established cell lines have implicated multiple oncogenes in animal cell culture models of neoplasia (2–4). These results herald a growing recognition among virologists that oncogenesis is a multistage process. The problem now is to identify the multiple stages in terms of specific genes and specific functions. As yet, neither the number nor the nature of essential functions required for tumorigenic transformation of rodent cells is known.

Superimposed on this hiatus in our knowledge is the question of the appropriateness of rodent cells as model systems in the study of human cancer (5). A close similarity between mouse and human neoplasia is shown, for example, by the comparison of chromosome translocations in Burkitt lymphoma, a rare human disease, and in mouse plasmacytomas (6). Both contain translocations that align the c-*myc* gene adjacent to immunoglobulin coding sequences. On the other hand, neither DNA nor RNA tumor viruses have been shown to play a role in human cancer comparable to that seen in rodents and birds (7, 8). Perhaps the most striking example of the resistance of human cells to viral transformation comes from the inadvertent inoculation of some 5,000–10,000 individuals with live simian virus 40 (SV40) present as a contaminant in early polio vaccine preparations (9). In the subsequent 20-odd years, not a single cancer has been reported as the result of this exposure (10). The manner in which herpesviruses (e.g., Epstein–Barr virus, cytomegalovirus) and the human T-cell leukemia (HTLV) retrovirus contribute to human neoplasias remains an elusive and complex problem (11–13). The resistance of human cells to tumor viruses is mirrored by their relative resistance compared with rodent cells to tumorigenesis after treatment with ionizing radiation (14) or chemical carcinogens (5, 15) in cell culture.

Another line of evidence, suggesting that human cells are more resistant to tumorigenesis than are rodent cells, comes from the stable nontumorigenicity of human cell hybrids produced by fusion between normal and tumor-derived (i.e., HeLa) cells of human origin (16, 17). Human cell hybrids of this type are much more stable than the analogous intraspecies cell hybrids of rodent origin. In the rare human cell hybrids that become tumorigenic, patterns of chromosome loss are seen that suggest the existence of tumor suppressor genes on particular chromosomes.

Given the powerful methods of recombinant DNA technology and DNA transfer into cells, it seems evident that the molecular identification of oncogenic DNA sequences should now be applied to human cells. Perhaps the heightened resistance of human cells to neoplastic transformation lies in an additional step in genetic control, in principle recognizable by available methods. One direct approach is to examine how human cells in culture respond to the cloned oncogenes that transform rodent cells.

The development in this laboratory of a strain of transfectable normal human diploid fibroblasts of foreskin origin called FS-2 has made it possible to compare the effects of cloned oncogenes introduced into human cells and into Chinese hamster CHEF/18 cells (18), which we previously showed to be transfectable (19). In this report, we describe the results of transfection into FS-2 cells of SV40 DNA and of the cloned *EJ* on-

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Abbreviations: SV40, simian virus 40; kb, kilobase(s); T antigen, tumor antigen.

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cogene derived from a human bladder carcinoma cell line (20). The *EJ* gene was identified and cloned from genomic DNA of the human tumor-derived cell line EJ by its ability to transform NIH/3T3 cells after transfection and later shown to hybridize with the retroviral oncogene v-Ha-*ras* (21–23). The Ha-*ras* gene codes for a 21-kilodalton protein, p21 (24) conserved in evolution but functioning in an as-yet-unknown manner. In the normal human p21, the 12th amino acid from the amino terminus is glycine, whereas in the various virus-encoded and *EJ* gene encoded p21s, other amino acids substitute for glycine (25–27). The altered protein is distinguishable in its electrophoretic mobility from normal p21 in transfected NIH/3T3 cells with a rat anti-p21 monoclonal antibody developed by Furth (28).

We report here that transfection of FS-2 cells with SV40 DNA (with a deletion in the late region) leads to morphological transformation but not to tumorigenicity. In contrast, transfection with the *EJ* gene has no apparent effect on the phenotype of FS-2, indicating that these cells are more resistant to transformation by the mutant c-Ha-*ras* gene than rodent embryo cells, which are partially transformed by this oncogene (2).

MATERIALS AND METHODS

Cells and Media. CHEF/18 cells were previously described (18). They are grown in α minimal essential medium (KC Biological, Lexexa, KS) with 5% fetal bovine serum (GIBCO). FS-2 cells were derived from a human foreskin and prepared in this laboratory by standard procedures. FS-2 cells were grown in Dulbecco's modified Eagle's medium (Flow Laboratories) with 10% fetal bovine serum. To test for anchorage independence, plates were prepared with 0.6% agar bases, and cells were added in suspension in 1.3% methylcellulose (Fisher) as described (18).

Vectors. Transforming DNAs were introduced in the following vectors. (*i*) λSV-9 is a cloned fragment in λgtWES from the SV40-transformed mouse cell line SVT2 containing an integrated copy of SV40 DNA deleted in the late region (29); (*ii*) pEJ contains the *EJ* gene as the 6.6-kilobase (kb) *Bam*HI fragment in plasmid pBR322 (20); (*iii*) pSVgpt and (*iv*) pSVneo (Bethesda Research Laboratories) are two similar shuttle vectors developed by Mulligan and Berg (30) into which we introduced the 6.6-kb *Bam*HI fragment containing the *EJ* gene. After transfection, cells containing λSV-9 or pEJ were selected on the basis of focus formation, whereas transfectants containing one of the shuttle vectors were selected either with mycophenolic acid at 25 μg/ml (*gpt* selection) or with the drug G418 (GIBCO) at 400 μg/ml (*neo* selection). Subsequently, drug-resistant cells were grown continuously in the presence of the selective drug.

Transfection. CHEF/18 cells were transfected essentially as described (19), except that cells were re-fed with fresh medium 4 hr before DNA was added. FS-2 cells were transfected according to the same protocol except that dimethyl sulfoxide treatment was omitted and 15% (vol/vol) glycerol was added for 4 min about 18 hr after DNA addition. The cells were then washed with Dulbecco's modified Eagle's medium and re-fed.

Restriction Site Analysis. DNA was prepared from drug-resistant FS-2 transfectants (gEF lines) essentially as described (31), except that ribonuclease digestion was carried out after the proteinase treatment to ensure removal of all RNA. Samples of DNA (5 μg) were digested to completion with *Sph* I or *Cla* I plus *Hin*dIII under reaction conditions recommended by the supplier (New England BioLabs). In double digestions the two restriction endonucleases were added together. DNAs were electrophoresed in 0.8% agarose gels and transferred to nitrocellulose filters as described (31). Filters were baked, prehybridized, hybridized to ^{32}P-labeled *EJ* (6.6-kb *Bam*HI fragment), washed, and exposed to x-ray film at −80°C in the presence of Du Pont Lightning Plus intensifying screen (31).

Identification of p21 Coded by *EJ* Gene in gEF Cells. Metabolic labeling of cells and p21 immunoprecipitations were carried out essentially as described (32) with minor modifications. Immunoprecipitation was performed with 10^7 cpm of trichloroacetic acid-precipitable material per sample and immunoprecipitates were subjected to electrophoresis in 5–15% linear gradient polyacrylamide slab gels. For immunoprecipitation the rat monoclonal antibody preparations 259, which reacts with both Ha-*ras* and Ki-*ras* gene products, and 238, which reacts specifically with the Ha-*ras* p21, were used (28).

RESULTS

Transformation of FS-2 Cells by Cloned SV40 DNA. Despite the evidence that SV40 is nontumorigenic in humans (9, 10), there are conflicting reports of its ability to transform human cells in culture (33, 34). To investigate this question, FS-2 cells were transfected with phage λSV-9 DNA. The cloned fragment of SV40 DNA is defective in the late region, preventing synthesis of viral proteins, but it retains transforming activity as shown by transformation of the Chinese hamster embryo fibroblastic cell line CHEF/18 (unpublished data).

In transfection experiments with FS-2 cells, monolayers (10^6 cells per dish) were treated with 20 μg of DNA per dish, either 1 μg of vector DNA plus 19 μg of carrier DNA (experimental) or carrier alone (control). Yields were 13 foci per dish in one experiment and 16 foci per dish in another, with no foci on dishes receiving only carrier DNA. Eleven foci were picked from separate dishes and grown for further testing. Each contained morphologically transformed cells similar to those shown in Fig. 1*C*. The transformed cells were shorter and plumper than normal FS-2 cells (Fig. 1*A*) and grew in a disoriented way, piling up and crisscrossing one another with a typical transformed appearance.

Genomic DNA from these transformed cells contained SV40

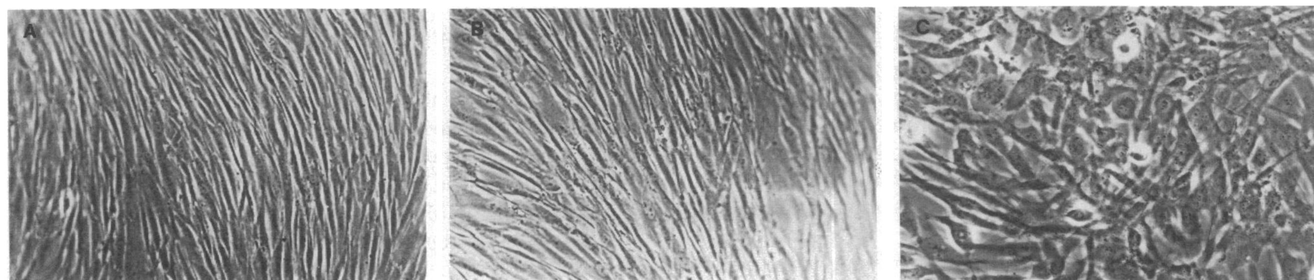

FIG. 1. Morphology of normal and transfected FS-2 cells. (*A*) Confluent monolayer of FS-2 cells showing typical spindle shape and parallel orientation of fibroblasts in culture. (*B*) Confluent monolayer of gEF4 cells containing integrated *EJ* gene. Morphology resembles that of FS-2. (*C*) Confluent SV-46 cells containing integrated defective SV40 DNA. Rounded cell morphology and crisscross orientation is typical of transformed cells.

Table 1. Transformation of CHEF/18 cells by vectors containing the *EJ* gene

DNA	Transformation, foci per pmol DNA
pEJ (linear)	550
pSVgptEJ (circular)	630
pSVneoEJ (linear)	1,140
Salmon sperm	<0.2

Five dishes with 10^6 cells per dish were treated with 1 μg of vector DNA plus 19 μg of salmon sperm DNA or 20 μg of salmon sperm DNA alone (control).

DNA as visualized in Southern blots of *Bam*HI-digested DNA hybridized with SV40 DNA. Four transfectants were studied further. All four produced T antigen in each cell as shown by indirect immunofluorescence microscopy, using anti-T antisera from hamsters and fluorescein-conjugated anti-hamster IgG (data not shown). The cells were not anchorage independent (plating efficiencies in methylcellulose less than 1% of the plating efficiency on plastic), and when each line was assayed for tumor-forming ability in *nude* mice (18) at four sites, 10^7 cells per site, no tumors arose during 6 months of observation.

These results are consistent with previous reports that human cells can be transformed in culture by SV40 but that the resulting transformants are not tumorigenic in the *nude* mouse assay (33). Since human cells are semipermissive for SV40, infected cells may undergo further damage after successive rounds of viral production and reinjection, complicating the analysis. The few instances of tumorigenic human SV40-transformed cell lines are probably of this type (34). We have used a defective SV40 DNA incapable of inducing virus production and thus the integrated viral DNA has been limited in its effect to coding for the T antigens (plus any consequences of the initial transfection and integration). In these circumstances, T antigens are seen to induce morphological transformation but not tumor-forming ability.

Transfection Studies with pEJ. The successful cloning of a DNA fragment from the human bladder carcinoma cell line (EJ) was made possible by its activity in transforming NIH/3T3 cells after transfection (1, 20–22). Availability of this cloned fragment, the *EJ* gene (20), permitted a direct test of the transforming ability of this gene for normal human cells. Our success in recovering foci after transfecting FS-2 cells with SV40 DNA showed that FS-2 cells were transfectable and that focus formation was an appropriate criterion for identifying transformation with this cell line. We further determined that the pEJ plasmid was effective in transforming CHEF/18 cells (Table 1). Thus, it seemed reasonable to anticipate focus formation after transfection with the *EJ* gene.

Five transfection experiments were performed. As shown in Table 2, only two foci were recovered on a total of 46 dishes

Table 2. Transfection of FS-2 cells by pEJ and λSV-9

	Transfection, total foci per total dishes		
Exp.	pEJ	Carrier DNA	λSV-9
1	2/8	0/9	—
2	0/9	0/3	26/9
3	0/6	0/3	3/5
4	0/3	—	20/5
5	0/20	0/10	30/9

pEJ or λSV-9 DNA was added at 1 μg per dish in the presence of carrier calf thymus or salmon sperm DNA at 20 μg per dish. As a control, carrier DNA was added alone at 20 μg/ml.

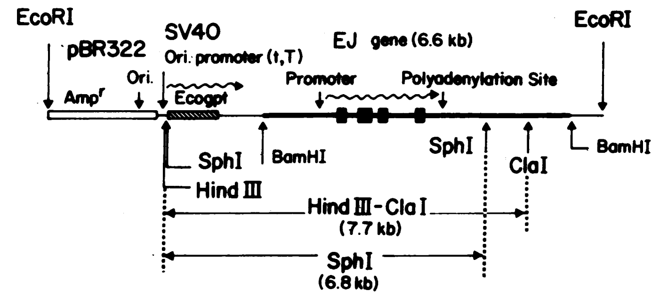

FIG. 2. Map of pSV2gptEJ vector (11.8 kb). Map shows position of restriction sites used in identification of integrated plasmid, as well as functions of components of the vector. Ori., origin of replication; Amp^r, ampicillin resistance. Map not drawn to scale.

each with 10^6 cells treated with pEJ DNA, whereas 79 foci were recovered on the positive control dishes treated with λSV-9 DNA and none on the negative controls treated with calf thymus or salmon sperm DNA. The two foci observed did not survive further culture.

This result demonstrated that the FS-2 cells had not produced foci in response to EJ DNA, whilst being successfully transfected by SV40 DNA in the same experiments. However, the experimental design did not provide a means for further analysis. Was the *EJ* DNA excluded or was it integrated but not expressed? To answer this question, it was necessary to monitor integration by introducing the *EJ* gene into a selectable vector. Two such vectors developed by Mulligan and Berg (30) became available for experiments of this kind.

Transfection of FS-2 Cells with Drug-Resistance Vectors. The cloned *EJ* gene was introduced into the pSVgpt and pSVneo vectors as shown in Fig. 2. To check the activities of the *EJ* gene in each vector construction, transfection into CHEF/18 cells was utilized. These experiments (Table 1) identified the biologically active vectors. In further studies it was shown that the transfected cells, selected by focus formation, were also drug resistant and tumorigenic (unpublished results).

The pSVgpt and pSVneo vectors containing the *EJ* gene were then utilized for transfection experiments with FS-2 recipient cells. Suitable conditions for drug selection were established: mycophenolic acid at 5–25 μg/ml for *gpt* selection and G418 at 400 μg/ml for *neo* selection. Drugs were added after allowing 4 days for expression. The results of these experiments are summarized in Table 3. Drug selection was essential for the recovery of FS-2 cells containing *EJ* DNA because no foci formed and the drug-resistant cells did not appear morphologically transformed. An example, gEF4 (*gpt-EJ*-transformed fibroblasts), is shown in Fig. 1*B*. Similar results were obtained after transfection with pSV2neoEJ.

Table 3. Formation of drug-resistant colonies after transfection of FS-2 cells with pSV2gptEJ or pSV2neoEJ

DNA	Transfection, colonies per pmol *EJ* DNA
pSV2gptEJ (circular)	36
pSV2gptEJ (linear)	172
pSV2neoEJ (circular)	64
pSV2neoEJ (linear)	133
Salmon sperm	0

Data are the results from two or more experiments, each with five or more dishes containing 5 or 10 × 10^5 cells treated with 1 μg of vector DNA plus 20 μg of salmon sperm DNA and five dishes with 20 μg of salmon sperm DNA alone.

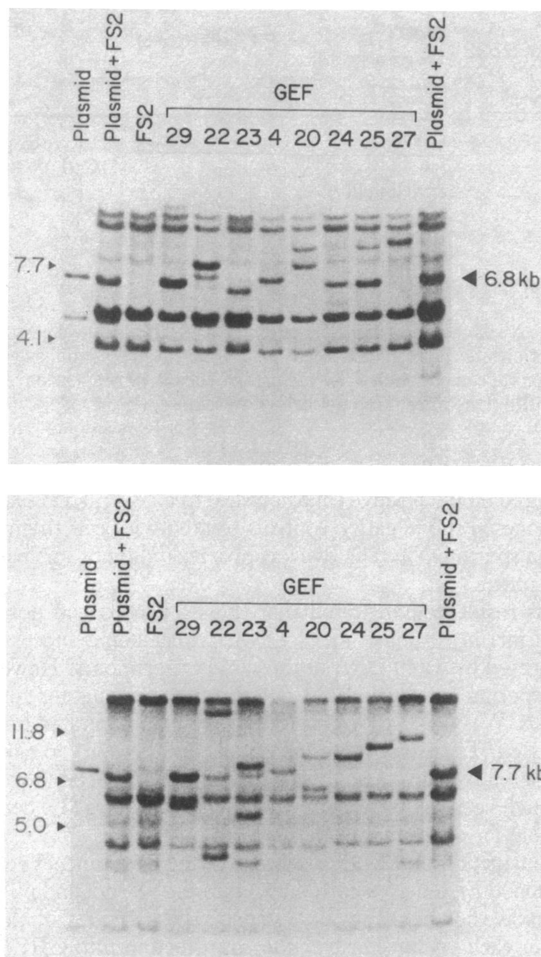

FIG. 3. (*Upper*) Integrated copies of *EJ* DNA in various gEF cell lines. Restriction fragments of genomic DNAs from gEF cell lines were cleaved by *Sph* I digestion, electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated ^{32}P-labeled 6.6-kb *Bam*HI fragment of pEJ as described (31). (*Lower*) Same DNAs as in *Upper*, digested with *Hin*dIII plus *Cla* I, processed as in *Upper*.

Presence of *EJ* DNA in gEF Cells. Thirty clones of gEF cells from foci of independent origin were examined for the presence of *EJ* DNA by Southern blot analysis. Eight candidate clones containing restriction fragments of appropriate size were chosen for further study. On the basis of a computer search of the *EJ* sequence (35), the *Sph* I and *Cla* I/*Hin*dIII restriction fragments (Fig. 2) were chosen as indicators of the presence of integrated fragments containing both the *Ecogpt* gene and the *EJ* gene.

As shown in Fig. 3 *Upper*, the unintegrated plasmid is cleaved into two fragments by *Sph* I, 6.8 and 5.0 kb. In the digest of FS-2 DNA mixed with plasmid DNA, these bands are seen to migrate slightly faster than in the lanes with plasmid DNA alone. Fragments corresponding to the 6.8-kb plasmid DNA are seen in five of the eight gEF cell DNAs: gEF29, -22, -4, -24, and -25. This fragment contains the entire *EJ* gene as well as the *Ecogpt* gene. In Fig. 3 *Lower*, the same DNAs are shown after digestion by *Cla* I plus *Hin*dIII. The plasmid DNA is cleaved to 7.7- and 4.1-kb fragments. The 7.7-kb fragment covers the same region as the *Sph* I 6.8-kb fragment but extends further into the 3' noncoding region of the *EJ* gene. Fragments corresponding to the 7.7-kb plasmid sequence are seen in gEF29, -22, -23, and -4, whereas larger novel bands are seen in the other gEF DNAs. Thus, gEF clones 4, 22, and 29 are the best candidates to contain the complete *EJ* genome since they contain bands of the correct size after cleavage both with *Sph* I and with *Cla* I plus *Hin*dIII. In addition it is likely that gEF24 and -25 also contain the complete *EJ* gene but that the plasmid was cleaved and integrated into genomic DNA in the region between *Sph* I and *Cla* I. These results leave open the possibility that clones 20, 23, and 27 may also contain a nonrearranged *EJ* gene. On the basis of visual inspection of the gels in Fig. 3, one can estimate that each of the transfected clones contained an average 1–2 copies of the transfected *EJ* DNA per cell.

Tumorigenicity of gEF Cells. The eight clones of gEF cells examined in Fig. 3 were tested for tumor-forming ability in the *nude* mouse assay. No tumors have developed in 4–6 months at a total of 25 sites each injected with $5–10 \times 10^6$ gEF cells (three or four sites per gEF clone).

Presence of p21 Protein in gEF Cells. The availability of anti-p21 monoclonal antibodies of rat origin (28) makes it possible to immunoprecipitate the p21 protein coded by the Ha-*ras* gene. In principle it should be possible to compare the amount and electrophoretic ability of the protein extracted from FS-2 cells, from EJ cells, and from the transfected gEF cells.

The results of preliminary studies with the eight gEF clones show that all but one contain a consistently higher amount of p21 protein than do FS-2 cells. Whether the extra protein is coded by the normal or by the mutant (*EJ*) gene is not yet clear. Unfortunately the difference in mobility of the normal and mutant human p21 proteins is more difficult to detect in human cell extracts than in mouse (25) or CHEF cell extracts, in which we have been able to identify the presence of *EJ*-encoded p21 readily (unpublished).

DISCUSSION

The responses of human diploid FS-2 cells of foreskin origin to transforming DNAs from the DNA tumor virus SV40 and from the human bladder carcinoma EJ (20) are compared in this paper. The DNAs were introduced by transfection (19).

FS-2 cells were morphologically transformed by SV40 DNA defective in the late region (29, 31). The cells produced T antigen but were not tumorigenic in the *nude* mouse assay. Thus, tumorigenicity was blocked despite the presence of T antigen, indicating that normal cellular gene products can override T-antigen tumor-inducing functions. This result is consistent with the vast epidemiological and clinical evidence that SV40 viral infection does not lead to cancer in humans (9, 10).

In contrast, FS-2 cells were not transformed by integrated copies of the mutant c-HA-*ras* gene—i.e., the *EJ* gene—cloned from the EJ cell line (20). The *EJ* gene was introduced both in the pEJ plasmid, leading to no observable transformation, and in the pSVgpt and pSVneo plasmids, which made possible selection of drug-resistant colonies composed of cells harboring the *EJ* gene intact but not expressed. No significant morphological differences from normal FS-2 cells were observed in more than 60 drug-resistant colonies that were grown up for further study. Twelve of the 30 clonal populations tested after pSVgptEJ transfection contained DNA hybridizing with the *EJ* 6.6-kb *Bam*HI fragment. Of the eight clones chosen for detailed analysis, five contained the *EJ* gene in restriction fragments of expected size, indicating intactness. Restriction fragment patterns for the other three clones did not exclude the presence of the intact gene. All eight were tested for tumor-forming ability in *nude* mice and all were negative.

In comparison with these results, NIH/3T3 cells transfected with the *EJ* gene are morphologically transformed and tumorigenic. When transfected into early passage fibroblasts from rat

embryo (2), the *EJ* gene induces morphological transformation and anchorage independence but not tumorigenicity. Thus, early-passage rodent cells not yet immortalized are susceptible to transformation in cell culture by the mutant c-Ha-*ras* gene but are not rendered tumorigenic. In contrast, the FS-2 cells, which are early-passage human fibroblasts, show no response to the presence of the *EJ* gene and are therefore distinct from early-passage rodent cells in their response to the mutant Ha-*ras* gene of human origin.

Our experiments provide a means to test whether the *EJ* gene fulfills Koch's postulates, according to which the "infectious agent" (*EJ* DNA) should be found in the diseased tissue (tumor), it should be isolated from the tumors, inoculated into fresh cells of the original kind, and again recovered from new tumors. Our procedure deviates from that prescribed by Koch in that we use human fibroblasts instead of bladder epithelial cells because media are not available for adequate growth of normal and transformed bladder epithelial cells. With this proviso, our results show that the *EJ* gene does not fulfill Koch's postulates.

In studies by other investigators of oncogene-induced tumorigenesis with rodent cell recipients, transformed cells in culture have been used instead of tumor-derived material as source of the DNA, and NIH/3T3 cells have been used instead of human cells for assay of biological activity. Koch's postulates have not been adequately tested in these systems, and the role of identified oncogenes in the origin of the human tumors from which they were recovered remains uncertain. Thus, it has not been demonstrated that the *EJ* gene was responsible for the bladder carcinoma from which the DNA was recovered or even that it played a role in the neoplastic genome.

In addition to these formal considerations, the fact that the EJ (or T24) cell line was isolated more than 10 years ago and has subsequently been propagated in culture raises the possibility that the nucleotide substitution present in this isolate may have arisen during cell culture. The fact that the *EJ* genome when first characterized was already highly aneuploid with many marker chromosomes (36) suggests that other DNA sequences with oncogenic potential were already present in the original tumor.

In summary, we have found that normal human fibroblasts are resistant to oncogenic transformation by SV40 DNA despite the presence of T antigen and are resistant to cellular transformation and tumorigenesis induced by the mutant c-Ha-*ras* gene from a human bladder carcinoma despite the presence of increased levels of p21 protein. These results are consistent with previous evidence of heightened resistance of human cells to experimentally induced oncogenesis compared with rodent cells. Since the development of cancer is strongly age related, special mechanisms of resistance to oncogenesis may have evolved in long-lived species. For example, the increased stability of the human chromosome complement in cells grown in culture compared with rodent cells may also be characteristic of the cells *in vivo* and thereby account for the appearance of human cancer on the average 50 years later than rodent neoplasias. Understanding the molecular basis of chromosome instability may provide new approaches to cancer prevention.

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