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Genetics

DNA transfer of focus- and tumor-forming ability into nontumorigenic CHEF cells

(transformation/human cancer/transfection/Chinese hamster cells)

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ABSTRACT **CHEF/18 fibroblastic cells derived from a Chinese hamster embryo are diploid and nontumorigenic and require multiple steps of chemical treatment and selection to produce tumorigenic derivatives. In this report, CHEF/18 cells and a mutant capable of growing in medium with a low concentration of serum, LS1-1, were recipients in DNA transfer experiments using the calcium phosphate coprecipitation method. Focus formation with donor DNAs from tumor-derived CHEF cells and from human bladder carcinoma cell line EJ gave yields of 0.02–0.59 focus per μg of DNA per 10^6 recipients. In one experiment in which CHEF/18 cells were transfected with EJ DNA, the presence of human DNA was detected in five of seven foci by using a cloned *Alu* sequence. Cells from one of these foci gave rise to tumors in *nude* mice, and the DNA produced secondary CHEF/18 transfectants. Because normal human cells as well as CHEF/18 cells require multiple stages to become tumorigenic, these findings suggest that EJ cells contain tumor-inducing DNA as the result of prior changes that occurred during the development of this carcinoma.**

The application of transfection methods (1, 2) to the transfer of purified DNA into mammalian cells in culture (3–6) has made possible a new experimental approach to investigating the genetic basis of malignant transformation (reviewed in ref. 7). With this method and the use of recombinant DNA technology, it should become possible to identify specific DNA sequences responsible for acquisition of tumor-forming ability.

A large body of information from the literature of carcinogenesis as well as clinical cancer points to malignancy as a multistep process that usually takes tens of years to develop (8). Although the immune system and other systemic mechanisms of protection may aid in slowing the development of the disease, it is evident that progressive cellular changes in genotype and phenotype are centrally important (9). In addition, many studies of the expression of malignancy in cell hybrids produced by fusions between normal and tumor cells have shown an initial suppression of tumor-forming ability (reviewed in refs. 10–12), suggesting that normal cells possess genes and gene products that block the expression of tumorigenicity.

Studies in other laboratories have shown by transfection experiments that DNAs of tumor origin can induce growth control changes—i.e., focus formation in recipient cells (13–19) and tumorigenicity (16, 17). In these studies, only the NIH/3T3 mouse fibroblast cell line has been used because of its ability to undergo DNA-mediated transformation at a considerably higher rate than other cell lines that were examined (7). Unfortunately, 3T3 cells have been shown to undergo spontaneous as well as chemically induced malignant transformation at rates indicating a single-step process (20, 21). Thus, with this system only a small window may be available for detection of potential oncogenes and the low number of tumor lines that have given positive results may be a consequence (7). As pointed out in a recent review, most cell lines are relatively refractory to transfected DNAs, and further uses of transfection may depend upon finding new recipient cell lines (7).

We report here that CHEF cells are good recipients for DNA transfer experiments, and that they can be used effectively to monitor the tumorigenic potential of tumor-derived DNAs. CHEF cells were developed in this laboratory for the genetic analysis of tumorigenicity (22–26). They are Chinese hamster fibroblastic cells of embryonic origin. The CHEF/18 line is nontumorigenic and diploid. No tumors have appeared in repeated tests in the *nude* mouse assay (22, 26). In a study of mutagen- and carcinogen-induced tumor formation (26) the frequency of tumors increased from 0/34 only to 3/43 after treatments in five separate experiments yielding 8–56% survival. After mutagenesis, we recovered 15 anchorage-independent mutants and 10 low-serum mutants (i.e., cells that grow with low concentrations of serum in the medium), only 5 of which were tumorigenic, despite the acquisition of transformed traits. A second round of mutagenesis led to tumorigenicity in some of these mutants. These studies have demonstrated the genetic stability of CHEF/18 cells and the multistep origin of tumorigenicity in this cell line. Chromosome analysis of these mutants has shown a high correlation of rearrangement with acquisition of tumor-forming ability (unpublished data).

In our initial DNA transfer experiments we used a low-serum mutant, LS1-1, as recipient because it is nontumorigenic but responds to chemical mutagens as if it were but a single step from tumorigenicity (26), somewhat analogous to 3T3 in this respect. Because of the extensive literature on the multistep origin of cancer, as well as our knowledge that CHEF/18 cells do not become tumorigenic in a single step, we considered it likely that DNA transfer experiments would have a greater chance of success with LS1-1 than with CHEF/18 as recipient.

We established a workable level of success with LS1-1, and then attempted similar experiments with CHEF/18 as recipient. In the experiments with CHEF/18 the donor DNA came from the human bladder carcinoma cell line EJ, which had previously been reported to be an effective donor in the transformation of 3T3 cells (16, 17). As described below, we have succeeded in recovering foci of transformed CHEF/18 cells that contain human DNA and are tumorigenic in the *nude* mouse assay. This result not only demonstrates that tumorigenicity can be transferred via DNA from cells of one species and histotype (human epithelial) to another (Chinese hamster embryo fibro-

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; TK, thymidine kinase; TE, Tris/EDTA; HAT, hypoxanthine/aminopterin/thymidine.

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blast) but also supports the hypothesis that the tumor-inducing DNA from the EJ cell line is the product of a multistep process by which the normal EJ cells became cancerous.

MATERIALS AND METHODS

Cell Lines. The origin and properties of CHEF/18, CHEF/16, and 205-30, a thioguanine-resistant CHEF/18 derivative, have been described (22). Briefly, CHEF/18 and CHEF/16 are diploid fibroblast cell lines derived from the same Chinese hamster male embryo and twice recloned. CHEF/18 cells are nontumorigenic (22, 26), whereas CHEF/16 cells are highly tumorigenic in the *nude* mouse (22). LS1-1 is a nontumorigenic derivative of CHEF/18 selected for growth in 1% fetal calf serum (26). 16-o is a hypoxanthine phosphoribosyltransferase-positive ($HPRT^+$), ouabain-resistant derivative of CHEF/16. 204-Tu-mtx is a tumor-derived methotrexate- and 5-bromodeoxyuridine-resistant CHEF/16 derivative. T21-4 is a tumor-derived line obtained after mutagenesis of CHEF/18 (26). EJ is a human bladder carcinoma line (27) obtained from Ian Summerhayes. Cells were grown as described (22) in α minimal essential medium (KC Biologicals, Lenexa, KS) with 5% or 10% fetal calf serum. All cell lines are mycoplasma-negative as judged by uridine/uracil incorporation ratios (28). For assays of tumorigenicity, congenitally athymic (*nude*) mice were injected subcutaneously in the flank with 4×10^6 cells (22).

Preparation of DNA for Transfection. Cells were treated with trypsin, centrifuged, and resuspended at 10^7 cells per ml in DNA buffer (0.1 M NaCl/0.02 M EDTA/0.5 M Tris·HCl, pH 8), and incubated 1 hr at 37°C with 0.5% sodium dodecyl sulfate and proteinase K (American Scientific Products, McGaw Park, IL) at 500 μg/ml. Sodium dodecyl sulfate was then added to 1% final concentration and the mixture was extracted twice with phenol. DNA was precipitated with 2 vol of cold absolute ethanol and resuspended overnight at 1/2 the original volume in TE (0.01 M Tris·HCl, pH 7.4/ 1 mM EDTA). RNase (Sigma) that had been boiled for 10 min was then added to 100 μg/ml for 1 hr at 37°C, after which proteinase K was added at 250 μg/ml for 30 min. The solution was extracted twice with phenol, twice with chloroform/isoamyl alcohol, 24:1 (vol/vol), and three times with diethyl ether. The aqueous phase was dialyzed overnight against 2 liters of TE, then NaCl was added to 200 mM and the DNA was precipitated with 2 vol of cold absolute ethanol. DNA was resuspended in TE and A_{260} and A_{280} were measured. Salmon sperm DNA (Sigma) was extracted twice with phenol, precipitated with ethanol, and resuspended in TE. Before use in transformation, DNA was sterilized by ethanol precipitation and resuspended in sterile 0.1 × TE.

Transformation. Recipient cells (10^6) were seeded in 100-mm dishes in 5% fetal calf serum/α minimal essential medium. After 24 hr 20 μg (1 ml) of DNA precipitate prepared by the method of Wigler *et al.* (4) was added per dish. Controls received no DNA. Dishes were incubated at 37°C for 16–18 hr (except experiment D, in which incubation was 8 hr). During the final 30 min, dimethyl sulfoxide was added to 9% (vol/vol) final concentration. Medium was then aspirated from the dishes, and the cells were washed once with α minimal essential medium salts and re-fed with medium containing 5% fetal calf serum. Approximately 48 hr was allowed for expression, after which cultures were either treated with trypsin and replated in selective medium or re-fed with selective medium. Cells were fed twice weekly and scored at 3–4 weeks.

Assay for Human DNA Sequences in Foci. Cells were harvested, and total cell DNA prepared as described (29). Approximately 10 μg of total cell DNA was digested with *Eco*RI (Bethesda Research Laboratories) or *Bam*HI (New England BioLabs). Reaction conditions were as recommended by the supplier. Agarose gel electrophoresis and transfer to nitrocellulose were as described (29). To detect human DNA, blots were hybridized to probes of cloned human *Alu* family DNA (30); BLUR 8 (31) was a gift from Robert Weinberg. This probe was ^{32}P labeled (10^7 cpm) by nick-translation and hybridized to blots of total cell DNA as described (29). Hybridized filters were exposed to x-ray film (Kodak XR-5) with an intensifying screen (Kodak X-Omat) at −80°C.

RESULTS

CHEF cells were shown to be good recipients for DNA-mediated gene transfer in preliminary experiments using cloned donor DNAs, as shown in Table 1. Cloned herpesvirus DNA containing the thymidine kinase (TK) gene gave rise to TK^+ transformants of TK^- CHEF/16 recipient cells. Phage λ clone 281-SV-1, containing one copy of a transformation-proficient but defective simian virus 40 DNA and flanking mouse sequences (32), produced foci when transfected into nontumorigenic CHEF/18 cells (Table 1).

Transfer of HPRT by Using CHEF Cell Genomic DNA. CHEF cells are derived from a male Chinese hamster embryo (22), and $HPRT^-$ mutants have been obtained at a frequency which suggests that only a single copy of the HPRT gene is present per diploid genome (unpublished data). Therefore successful transfer of the HPRT gene to $HPRT^-$ CHEF cells by using unfractionated CHEF cell donor DNA should show the feasibility of using CHEF cells in assays of other single-copy genes—e.g., putative oncogenes. In order to use the same DNA for transfer of both HPRT and putative oncogenes, donor DNA for HPRT experiments was prepared from an $HPRT^+$ tumorigenic CHEF/16 line, 16-o. Fourteen cultures of nontumorigenic $HPRT^-$ 205-30 recipient cells were incubated with 16-o DNA. Thirteen parallel control cultures did not receive DNA. After 54 hr in nonselective medium each dish was treated with trypsin, and the cells were replated into seven dishes in hypoxanthine/aminopterin/thymidine (HAT) selective medium (Table 2). Replating was performed to allow the number of population doublings required for killing of $HPRT^-$ cells by HAT. Although the expression time allowed should have been sufficient for two or three population doublings, hemocytometer counts at the time of replating indicated that only one population doubling had occurred in DNA-treated cultures.

A total of nine $HPRT^+$ colonies appeared on the DNA-treated dishes, arising from eight different original DNA-treated cultures (Table 1). One colony arose on 1 of the total of 91 control dishes (replated from 13 original control plates).

Table 1. DNA transfer using cloned donor DNA*

Exp.	DNA	Total foci or colonies/ total dishes	Average foci or colonies per original dish†	Foci or colonies per μg DNA‡ per 10^6 cells
1	281-SV-1	≈1,000/10	200	20
	None	16/10	3.2	—
2	Herpesvirus TK gene	129/39	25.8	198
	None	3/40	0.6	—

* The recipient cells were CHEF/18 $HPRT^-$ in Exp. 1 and CHEF/16 TK^- in Exp. 2.

† Five dishes split to 10 after 48-hr expression time in Exp. 1. Five dishes split to 40 after 48-hr expression time in Exp. 2.

‡ Fifty percent of recombinant phage is simian virus 40 DNA; 20 μg per dish used in Exp. 1. Thirteen percent of recombinant plasmid is TK gene; 1 μg/dish used in Exp. 2 (plus 19 μg of salmon sperm DNA).

Table 2. DNA transfer of HAT resistance to HPRT$^-$ cells (line 205-30)

DNA	Positive original cultures	Total colonies/ total replated dishes	Average colonies per original culture	Colonies per μg DNA per 10^6 cells
16-o (HPRT$^+$)	8/14	9/98	0.64	0.03
None	1/13	1/91	0.08	—

Because eight of nine original DNA-treated dishes each gave rise to only one HPRT$^+$ colony on replating, we conclude that essentially no doublings of DNA-transformed cells had occurred prior to replating. Thus, replating to optimize expression and selection does not artificially increase the yield.

Transformation of LS1-1 by Human and CHEF DNAs. LS1-1 recipient cells were exposed to DNA from a human bladder carcinoma line (EJ), tumorigenic CHEF/16 cells (16-o), and tumor-derived CHEF cells (T21-4 and 204-Tu) (Table 3). DNA precipitates were prepared by the method of Wigler *et al.* (4) except in experiment D, in which the DNA was dissolved in 280 mM NaCl/50 mM Hepes/1.5 mM sodium phosphate, pH 7.1, and $CaCl_2$ was added as a final step. After 48-hr expression time DNA-treated and control cells were shifted to medium containing 3% fetal calf serum (experiment B) or treated with trypsin and replated into two (experiment D) or three (experiment C) dishes with 3% fetal calf serum medium. Reconstruction experiments have shown that this reduction in serum concentration enhances the differences between the growth patterns of transformed and nontransformed cells.

Both tumorigenic and tumor-derived hamster DNAs gave rise to foci on LS1-1, with yields that ranged between 0.02 and 0.18 focus per μg of DNA per 10^6 recipients. Human bladder carcinoma DNA gave the highest yield of foci, 0.59 focus per μg of DNA per 10^6 recipients. Within each experiment, the number of foci on DNA plates was at least twice that on control plates. Neither salmon sperm DNA nor LS1-1 DNA gave a significant increase in focus formation over background.

With 204-Tu-mtx donor DNA, it was observed that replating into selective media (experiment C) gave an 8-fold increase in foci per μg of DNA over merely refeeding without replating (experiment B). Hemocytometer counts at the time of replating indicated that DNA-treated cells had undergone only one population doubling during the expression time. Further, as shown in Table 2, no increase in colony number of transformants was seen after replating. Therefore, growth of DNA-transformed cells during the expression time appears not to account for the increased number of foci observed with replating.

Experiments C and D both involved replating, but with 204-Tu-mtx DNA the yield of foci per μg of DNA was 4-fold higher in experiment C than in D. The increased yield observed in C is the result of longer incubation with DNA (16 vs 8 hr) and of the smaller size and more even spreading of DNA precipitate prepared by the method of Wigler *et al.* (4) (experiment C) over an alternative method (experiment D).

Table 4. Focus formation by CHEF/18 cells induced by transfer of DNA from the human tumor bladder carcinoma EJ

DNA	Total foci/ total dishes	Average foci per original dish*	Foci per μg DNA per 10^6 cells (background subtracted)
None	41/16	2.2	—
EJ	74/14	5.2	0.15

* Some cultures were split after incubation with DNA to facilitate focus formation, as in Table 3.

Transformation of CHEF/18 by Human Tumor DNA. CHEF/18 cells were incubated with DNA of the human bladder carcinoma line EJ in medium with 5% fetal calf serum. After 80-hr expression time, cells were shifted to medium containing 3% fetal calf serum for focus selection. A typical yield from an experiment using CHEF/18 cells as recipients is shown in Table 4. Seven foci were picked and DNA was prepared, run on agarose gels, transferred to nitrocellulose paper, and hybridized with a nick-translated clone of the human *Alu* family of repetitive interspersed sequences (30, 31). This probe identifies human DNA sequences present in high molecular weight DNA from the transformed hamster cells. As can be seen in Fig. 1, five of the seven foci showed hybridization to the *Alu* probe,

Table 3. Transformation of LS1-1 by tumor DNAs

Exp.	DNA	Fraction positive cultures	Total foci/ total dishes	Average foci per original dish*	Average foci per original dish minus background	Foci per μg DNA per 10^6 cells
A	None	7/16	9/16	0.6	—	—
(no replating)	Salmon sperm	5/10	7/10	0.7	0.1	0.005
	LS1-1	4/10	6/10	0.6	0	<0.005
B	None	3/11	3/11	0.27	—	—
(no replating)	204-Tu-mtx	12/19	13/19	0.68	0.41	0.02
	T21-4	6/10	10/10	1.00	0.73	0.04
C	None	8/14	26/32	1.8	—	—
(replating in	204-Tu-mtx	7/10	49/27	5.5	3.7	0.18
three plates)	EJ	10/10	125/25	13.6	11.8	0.59
D†	None	0/11	0/18	<0.06	—	—
(replating in	204-Tu-mtx	3/9	6/16	0.78	0.78	0.04
two plates)	16-o	4/10	7/19	0.90	0.90	0.05

* In Exps. C and D this number is calculated for any dishes lost during incubation: average foci per surviving replated dish is multiplied by the number of dishes into which the cells in each original dish were replated, and the total is divided by the number of original DNA-treated cultures.

† DNA in Exp. D prepared by different procedure than in Exps. A, B, and C (see *Materials and Methods*).

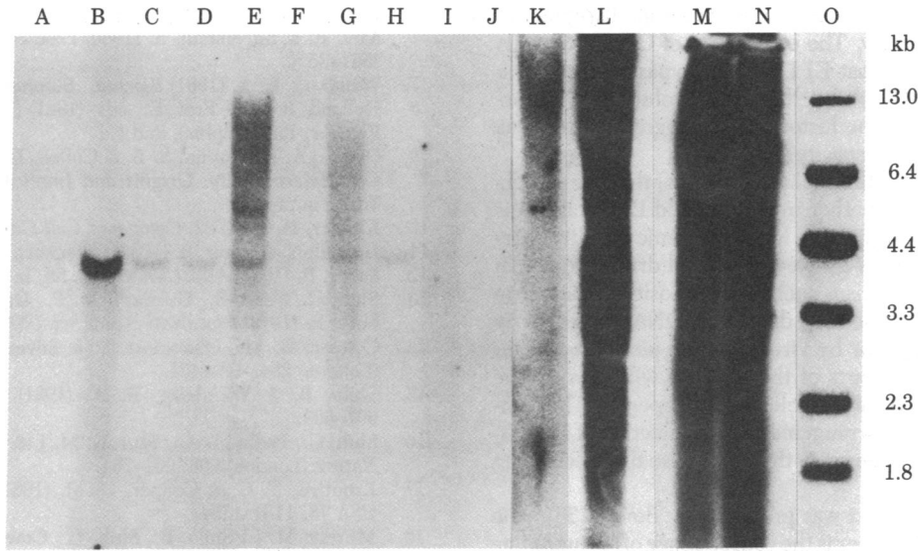

FIG. 1. Blot hybridization of the *Alu* (BLUR-8) probe to DNA from CHEF/18 cells transfected with EJ DNA and from two tumors derived from primary transfectants. Genomic DNAs were extracted, digested with *Bam*HI, electrophoresed on 1% agarose, transferred to nitrocellulose paper, and hybridized to ^{32}P-labeled nick-translated (10^7 cpm) BLUR-8 DNA. Positive lanes are B, E, G, I, and K, containing genomic DNA from foci 29-1, 29-5, 29-11, 29-13, and 29-10, respectively, and M and N from tumors derived from injection of line 29-5 into *nude* mice (4×10^6 cells per site). Lane L contains genomic DNA from cell line EJ. Negative lanes are A, C, D, F, H, and J, containing genomic DNA from CHEF/18, 29-1-2, 29-2, 29-7, 29-11-4, and LS1-1, respectively. The band at 4.2 kb may represent hamster reiterated sequences that cross-hybridize to the human *Alu* sequence. Standards (lane O) are pBR322 and another pBR-containing clone. Film showing lanes A–J was exposed 6 days; lanes K–O were exposed 1 day.

as shown by an intense band (f-29-1) or broad smears of hybridizing DNA resulting presumably from multiple sites of integration. The pale band at 4.2 kilobases represents Chinese hamster DNA that hybridized to the probe. These results demonstrate that acquisition of the ability to form foci after exposure to human tumor DNA paralleled acquisition of human DNA sequences.

Cells from the positive focus, f-29-5, have given rise to tumors in 5 weeks. DNA from two of these tumors was examined by cleavage with *Bam*HI, electrophoresis on agarose, transfer, and blot hybridization with the *Alu* probe. As shown in Fig. 1, lanes M and N, both tumors contained multiple sequences of human DNA, heavily enriched over that seen in the original f-29-5 cells. In addition, DNA from f-29-5 cells has been used in a second-generation experiment and has again induced focus formation in CHEF/18 cells.

DISCUSSION

This paper describes the use of CHEF cells as recipients for DNA transfer experiments. The CHEF/18 cell line is diploid ($2n = 22$), it is nontumorigenic, and treatments with carcinogens have demonstrated the stability of its genome and the rarity of tumorigenic cells arising after chemical carcinogenesis (22–26). CHEF/18 cells, like normal human cells, do not become tumorigenic in a single step, and consequently this cell line is particularly valuable for studies of the genetic basis of tumorigenicity.

Focus formation in the range of 0.02–0.05 focus per μg of DNA per 10^6 cells above background was found with three different tumorigenic donor DNAs of CHEF origin, two of them tumor derived. One of these DNAs, from 204-Tu-mtx, a methotrexate-resistant tumor-derived line, was used in a series of transformations (Table 3) designed to find out whether expression could be enhanced by replating the recipient cells 48 hr after DNA treatment. An increased yield from 0.02 for unreplated cells to 0.18 focus per μg of DNA per 10^6 cells resulted from splitting the cells 1:3 when they were replated. This increase probably does not result from cell division of transformed cells, because total plate counts of DNA-treated cells only doubled in this period, and no increase in yield was found with HPRT transformation (Table 2). We think that the additional growth made possible by replating may enhance focus formation. The yield of foci on dishes treated with EJ DNA was 3 times that with 204-Tu-mtx DNA, and both were in the range described for 3T3 cells (13–19). Additional work is needed to maximize transformation frequencies with our cells, but the yields reported here are adequate for many types of further experiments.

Evidence that transforming DNA is responsible for the observed change in recipient phenotype—i.e., focus formation or colony formation or colony growth in the presence of selective drugs—can be adduced in several ways. Most important is the demonstration that DNA of donor origin has actually been incorporated into the recipient genome. For this purpose we have used cloned human DNA containing the *Alu* sequence that is relatively specific to human DNA (30, 31). We hybridized a nick-translated BLUR-8 DNA to total genomic DNA of cells grown from foci induced by treatment of CHEF/18 cells with EJ DNA. As shown in Fig. 1, five of the seven foci so far tested have been positive, demonstrating that these cells contain integrated and replicated copies of human DNA. Identification of particular sequences associated with tumor-forming ability awaits the development of appropriate cloned sequences. Fulfillment of Koch's postulates will be achieved when cloned sequences are identified not only in focus-forming cells but, most important, also in cells derived from tumors produced by recipient transformed cells and used successfully in further rounds of transformation.

The experiments reported here have shown that stable nontumorigenic CHEF/18 cells can be induced to form foci and make tumors in *nude* mice by treatment with EJ DNA derived from a human bladder carcinoma cell line (27), as can LS1-1

cells, which grow in 1% serum and may be intermediates on the pathway to tumorigenicity. The conversion of CHEF/18 cells to tumorigenicity shows that EJ DNA can bypass the multiple steps required by CHEF/18 cells in the evolution of tumor-forming ability. What is the history of EJ progression that has led to acquisition of this capacity?

It is our working hypothesis (11, 12, 33–35) that the tumorigenic potential of EJ and other tumor-derived DNAs arises as a multistep process, in which the loss of genomic stability characteristic of normal cells is the key event and driving force. In this view, once a mechanism such as transposition leading to genomic instability is induced by damage to DNA—whether by chemicals, by radiation, or by viruses—then new associations of DNA from different parts of the genome will provide the heterogeneity on which natural selection can operate. The combination of continuing rearrangements and selection of increasingly successful phenotypes may then comprise the mechanism of stepwise progression.

A hypothesis of this sort was proposed by Boveri (36), who argued brilliantly that cancer is the consequence of chromosome abnormalities. Support for this general view has come especially from the growing evidence of nonrandom translocations and other chromosome changes associated with particular forms of clinical cancer, including leukemias (37), lymphomas (38), and meningiomas (39). Cells with specific translocations are thought to overgrow the population by natural selection from the mass of nonspecifically changed cells seen during evolution of the disease; these translocations may represent the linking of two or more DNA sequences that together overcome growth control and permit proliferation (9).

Our evidence that EJ DNA can convert CHEF/18 cells to tumorigenicity in a single step is consistent with this hypothesis. The idea may soon be testable that damage to DNA can lead to cancer by destabilization of the genome and activation of rearrangement mechanisms.

Note Added in Proof. DNA extracted from LS1-1 cells transfected with EJ DNA (Table 3) has been found to contain human DNA that hybridizes with the BLUR-8 probe in 8 out of 11 foci tested.

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