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Passage of phenotypes of chemically transformed cells via transfection of DNA and chromatin

(chemical carcinogenesis/transformation alleles/Southern blotting)

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ABSTRACT **DNA was prepared from 15 different mouse and rat cell lines transformed by chemical carcinogens *in vitro* and *in vivo*. These DNAs were applied to NIH3T3 mouse fibroblast cultures by using the calcium phosphate transfection technique. DNAs of five donor lines were able to induce foci on the recipient monolayers. Ten other donor DNAs yielded few or no foci. DNAs from control, nontransformed parental cell lines induced few or no foci. Chromosomes were transfected from one donor whose naked DNA was unable to induce foci, and morphologic transformation of recipients was observed. These experiments prove that in five of these cell lines the chemically induced phenotype is encoded in DNA, and the sequences specifying the transformed phenotype behave as a dominant allele in the NIH3T3 recipient cells. The sequences encoding the transformation are likely found on a single fragment of DNA.**

The molecular mechanisms of chemical carcinogenesis are poorly understood. Work of Ames and others (1–4) has demonstrated a strong correlation between the mutagenicity and carcinogenicity of a large series of compounds, suggesting that DNA is the ultimate target of the carcinogens. Experiments of others (5–7) have shown that the rate of focal transformation elicited by chemical carcinogens on monolayer cultures *in vitro* occurs with an efficiency within an order of magnitude of the efficiency of mutagenesis of a specific marker gene carried by these cells. Taken together these experiments might suggest that the mutation of one of several target genes in these cells leads to transformation and ultimately to tumorigenicity. Nevertheless, there has been no direct proof that the chemically induced transformation phenotype is encoded within the DNA and that the phenotype is specified by a discrete segment of genetic information.

We report here experiments designed to investigate the transmissibility of the chemically transformed phenotype from cell to cell via purified DNA. This demonstration depends upon the transfection technique of Graham and van der Eb (8) in which DNA extracted from donor cells is introduced into recipients as a coprecipitate with calcium phosphate. Previous work in this laboratory utilized this technique to demonstrate the infectivity of several forms of murine leukemia virus DNA (9, 10). More recently, this technique was applied to demonstrate the biological activity of *in vitro* synthesized, subgenomic fragments of murine sarcoma virus (MSV) DNA (11) and of several forms of *in vivo* synthesized Harvey MSV (unpublished results). In addition, work of others has demonstrated the transmissibility of other viral and cellular genes via this technique (12, 13).

The transfection of these sarcoma virus DNAs led to the observation of foci of transformed cells whose behavior was indistinguishable in many cases from that of virus-infected cells. Some of these transfections utilized donor cellular DNA in which the transforming genome was present in single copy number per haploid cell DNA complement. We reasoned that nonviral transforming genes, if present in unique copy number, might also be transferable via DNA transfection. Specifically, we attempted to demonstrate the existence of genes in the DNA of chemically transformed cells whose introduction into normal recipients would result in focal transformation of the recipient monolayer.

MATERIALS AND METHODS

Cell lines used here are described in Table 1. DNA transfection procedures were as described (11). DNA was prepared from tumors or cell lines as described (23).

Chromatin transfection procedures were as described (24) with the following exceptions: (*a*) recipients were not pretreated with mixtures of colchicine, Colcemid, and cytochalasin D before transfection, (*b*) gentamicin was not used in these experiments, (*c*) instead of counting the chromosome number under a microscope, quantitation of chromosomes was done by spectrophotometric absorbance, and (*d*) 10% dimethyl sulfoxide posttransfectional treatment was not always included.

Southern gel-filter transfer was performed as described (12). The soft agar assay was done by pouring 0.3% soft agar (Difco) containing 3000 cells over a 0.6% agar layer in a 6-cm dish. Colonies were scored 14 days later. Transforming virus rescue assays were done as described (11).

RESULTS

A series of 15 different cell lines (Table 1) were collected from various sources. These cell lines were all of murine origin and most were transformed *in vitro* by various carcinogens commonly used for *in vivo* and *in vitro* chemical carcinogenesis (25, 26). DNA from all these lines was prepared and transfected in a fashion identical to that used in the transfection of retrovirus DNAs. The recipient cells used for monitoring the biological activity of these DNAs were a subline of NIH3T3 cells which fulfills two requirements for these experiments. First, these cells take up DNA in a biologically active form at high efficiency compared with most other mouse cell lines that we have tested. Second, these cells are contact inhibited, and the monolayers they form allow relatively easy visualization of transformed foci.

After transfection of NIH3T3 cultures, the cells were reseeded and scored for foci 14–20 days after transfection. The scored foci were examined individually and were counted only if the constituent cells were hyperrefractile, grew in a criss-crossed pattern, and formed a colonial morphology that we feel

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Abbreviation: MSV, murine sarcoma virus.

Table 1. Origins of cell lines used in transfections

Cell line or *in vivo* dissected tumor (ref.)	Induced by[a]	Parental cell line or animal
MC5-5 [Y. Ikawa (14)]	3-MC	BALB 3T3
DMBA-BALB 3T3 (15)	DMBA	BALB 3T3
MCA16 (16)	3-MC	C3H10T1/2
MCA5 (16)	3-MC	C3H10T1/2
MB66 MCA ad 36[b]	3-MC	C3H10T1/2
MB66 MCA ACL 6[b]	3-MC	C3H10T1/2
MB66 MCA ACL 13[b]	3-MC	C3H10T1/2
MB66 MCA ACL 14[b]	3-MC	C3H10T1/2
MC-1 (17)	3-MC	C3H10T1/2
TU-2 (17)	UV	C3H10T1/2
F-17 (18)	X-ray	C3H10T1/2
F-2407-NQO c11W (19)	NQO	F-2407
BP-1 fibrosarcoma[c]	BP	C57BL × C3H/HeJ
BP-2 fibrosarcoma[c]	BP	C57BL × C3H/HeJ
BP-3 fibrosarcoma[c]	BP	C57BL × C3H/HeJ
C3H10T1/2 (20)		
NIH3T3 (21)		
BALB 3T3 (22)		

[a] 3-MC, 3-methylcholanthrene; DMBA, 7,12-dimethylbenzanthracene; NQO, 4-nitroquinolene-1-oxide; BP, benzo[*a*]pyrene.
[b] Gifts from U. Rapp.
[c] Animals with tumors were provided by P. Donahue and G. N. Wogan.

is representative of a true transformed colony (Fig. 1). Nevertheless, control nontransfected monolayers and monolayers transfected with control nontransformed donor DNAs occasionally exhibited spontaneous foci, a few of which were not readily distinguishable from true transformants. Therefore, unless otherwise indicated, all focus counts presented here were the results of double-blind experiments. After preparation of DNAs from transformed and nontransformed control cultures, the DNA samples were encoded before transfection. Several days before final evaluation of foci, each of the culture dishes was encoded a second time and the experimental and control cultures were randomized. After the foci in the dishes were counted, the identities of the cultures were decoded and the data were tabulated. We believe that this procedure would neutralize the effects of subjective evaluations of focal morphologies.

Transfection of DNA of Chemically Transformed Clones. It was soon apparent that the donor DNAs could be grouped into two classes. The first class consisted of cells whose DNAs yielded none or a few (1 or 2) distinctive foci after transfection of 75 μg of DNA onto 1.5×10^6 cells (Table 3). Although this small number of foci seen upon transfection was quite distinctive and differentiable from spontaneous overgrowths, the number was so small and irreproducible that we do not presently regard these results as credible.

A second group of donor DNAs reproducibly yielded foci with high efficiency. Representative double-blind experiments to evaluate one of these high-efficiency donor DNAs are summarized in Table 2. This group consists of five lines derived from independently transformed foci of C3H10T1/2 cells and another line of BALB 3T3 origin. These high-efficiency DNAs yielded foci at a rate of 0.1–0.2 focus per μg of transfecting cellular DNA, a transfection efficiency comparable to that observed upon transfection of integrated retrovirus genomes present in low copy number in cellular DNA. In order to assure identity of two of these cell lines, independent aliquots of each were received from U. Rapp and C. Heidelberger 6–12 months after receipt of initial samples. The subsequently received cultures yielded DNA that behaved identically to their previously characterized counterparts. Further controls showed that the transmissibility of the transforming alleles is resistant to ribonuclease treatment and is destroyed by some but not by all site-specific DNA endonucleases (unpublished results).

Transfection with Chromatin of Chemically Transformed Cells. The failure to rigorously demonstrate a transmissible transforming gene in the DNAs of some cell lines listed in Table 1 could be attributable to the absence of a discrete, transmissible allele in these cells. Alternatively, the transforming alleles of these cells might well be in a configuration that allows them to exhibit only relatively low transfection efficiency. This latter possibility was plausible because our previous transfection of retrovirus DNA indicated that the presence of certain linked sequences could affect the transfection efficiency of the Moloney MSV transforming gene by as much as two orders of magnitude (11).

Work of others (27–30) had demonstrated that the transfection of chromosomes rather than of naked DNA allowed a considerable enhancement in transfection efficiency (24). Therefore, we attempted transfection of metaphase chromosomes from a dimethylbenzanthracene-transformed BALB 3T3 cell line. The DNA of these cells had previously yielded a small, irreproducible number of foci (0–2 per 75 μg of DNA). As a control, we transfected chromosomes from a cell line (MC5-5)

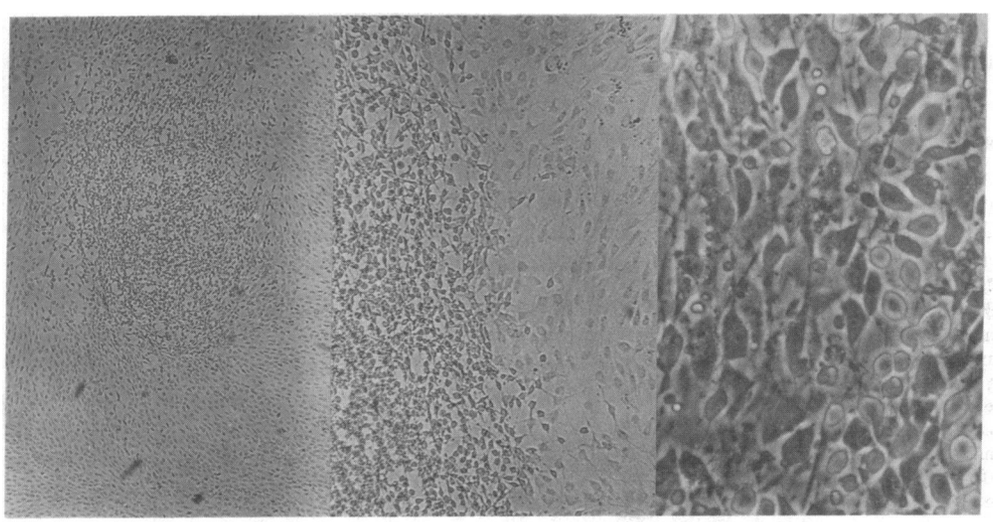

FIG. 1. Foci of transfectants at different magnifications. (*Left*) DNA of MCA16 yielded focus MCA16-5 whose DNA was in turn transfected to yield MCA16-5-1, pictured here. (*Center*) DNA of MCA5 was used to derive focus MCA5-1, pictured here (the focus is seen to the right of the frame). (*Right*) DNA of MC5-5 was used to derive focus MC5-5-4, pictured here.

Table 2. Double-blind evaluations of foci after DNA transfections[a]

Experiment	Donor cells	Foci in individual culture dishes	Total foci per experiment
I	MC5-5-0	6, 3, 4, 3, 6, 9, 5, 2, 4, 6[b]	48
	NIH3T3	0, 0, 0, 0, 0, 0, 0, ≤1, 0, 0, 0, 0	≤1
II	MCA16	2, 2, 0, 0, 0, 1, 0, 0, 0, 0, 0, 0	5
	MB66 MCA ad 36	0, 1, 2, 0, 2, 0, 0, 1, 0, 0, 1, 1	8
	MB66 MCA ACL 6	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
	MB66 MCA ACL 13	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0
	C3H10T1/2	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0

[a] DNA (75 μg) was transfected onto 1.5×10^6 NIH3T3 cells, which were reseeded into 12 100-mm dishes 4–6 hr posttransfection. The culture dishes were encoded and randomized and foci were counted 14–18 days later.
[b] Two cultures were lost due to contamination.

whose naked DNA had previously yielded foci with reasonable efficiency. As seen in Table 3, transfection of these chromatin preparations yielded, via double-blind experiments, a significant level of foci, whereas transfection of chromatin of the BALB 3T3 parent cell line yielded a low background level of foci.

Serial Passaging of Transformed Allele. In order to determine whether these alleles could be passaged serially, one focus (termed MCA16-5) was picked after transfection of DNA from the MCA16 donor. A second focus (termed MC5-5-6) was picked after transfection of the chromosomes of the MC5-5 line. These cells were subjected to single-cell cloning. Their DNA was prepared and further tested for biological activity. As seen in Table 4, both DNAs demonstrated high levels of biological activity (10–375 foci per 75 μg of DNA). Therefore, the transforming element of transformed C3H10T1/2 and BALB 3T3 cells is passageable from donor to recipient over two cycles of transfection. More recent work demonstrates the transmissibility of several of these alleles through a third serial cycle of transfection.

Involvement of Retrovirus Genomes in Transformation. It was possible that the transforming genes detected here reflected adventitious laboratory contamination of cultures by Harvey or Moloney MSVs, both of which are used in this laboratory. Alternatively, an endogenous retrovirus genome might have been activated in these cells (16, 31, 32). We attempted to minimize this possibility by demonstrating the absence of transmissible type C retrovirus transforming genomes. Although these transforming viral genomes are normally replication defective, they can be transmitted by superinfection of virus-

Table 3. Characterization of donor and transformants used in transfection

Donor DNA[a] or chromosomes prepared from	Transfection efficiency	Growth in agar[b] of		Rescue of transforming virus[g] from	
		Donor	Derived transformants	Donor	Transformants
Naked DNA transfections[c]					
BALB 3T3	1, 1, 0	−		−	
MC5-5	6[d], 6[d], 2	+++; >25%		−	
C3H10T1/2	1, 1, 0			−	
MCA 5	9–14[d], 8, 6, 5	++; 70%	+; 1–10%	−	−
MCA16	12[d], 7–10[d], 5, 3		++; 25%	−	−
MB66 MCA ad 36	8, 15	ND	ND	ND	ND
MC-1	10	ND	ND	ND	ND
Other controls					
NIH3T3	0, 0[d]	−		−	
NSF mouse liver	2[d], 1, 0				
MSV-Transformed NIH3T3	10[d], 100[d]	+	+	+	+
Mock[e]	2, 0				
Chromosome transfections[f]					
BALB 3T3	1, 1–2	−		−	
MC-5	20[d], 30	+++; >25%	+; 2–15%	−	−
DMBA-BALB 3T3	15[d]	±; <0.1%	−	−	−

The following lines gave 0–2 foci per 76 μg of DNA: MB66 MCA ACL 6, MB66 MCA ACL 13, MB66, MCA ACL 14, TU-2, F-17, BP-1, BP-2, BP-3, F-2407-NQO c11W, DMBA-BALB 3T3. ND, not done.
[a] These are described further in Table 1.
[b] Symbols: +++ and ++, macroscopic-sized colony; +, regular size colony; ±, small colonies; −, no colonies seen. Percentages represent plating efficiencies in soft agar.
[c] Number of foci per 75 μg of DNA per 1.5×10^6 transfected NIH3T3 cells.
[d] Not performed in double-blind experiment.
[e] Mock, no DNA added to calcium phosphate precipitate.
[f] Number of foci after transfection of chromosomes containing 75 μg of DNA applied to 1.5×10^6 NIH3T3 cells.
[g] Symbols: +, $>10^5$ focus-forming units of transforming virus rescued per ml; −, no transforming virus rescued; ND, not done. All murine leukemia virus-infected cells released $>10^6$ plaque-forming units of murine leukemia virus per ml.

Table 4. Serial passage of transformed phenotype

Primary transfection	Secondary transfection
MC5-5 —(20 foci/75 μg DNA, chromosomes)→ MC5-5-6	—(28 foci/75 μg DNA, naked DNA)→ MC5-5-6-1
MCA16 —(5, 12[a] foci/75 μg DNA, naked DNA)→ MCA16-5	—(10, 375[a] foci/75 μg DNA, naked DNA)→ MCA16-5-1

[a] The two numbers represent two independent DNA preparations.

transformed cells with a replication-competent, nontransforming murine leukemia virus whose genome allows pseudotyping and transmission of the transforming MSV genome. As seen in Table 3, whereas murine leukemia virus superinfection of an MSV-transformed cell line results in release of $>10^5$ focus-forming units of MSV per ml and $>10^6$ plaque-forming units of murine leukemia virus per ml, superinfection of a series of donor and derived transformant cell lines yielded high levels of the superinfecting murine leukemia virus but no rescue of retrovirus transforming genomes. Although we cannot presently exclude the intervention of various other viral genomes in this transformation, transmissible type-C retrovirus genomes do not appear to be responsible for the observed phenomena.

Genetic Background of Donor and Recipient Cell Lines. Additional control experiments were designed to rule out the possibility that the foci of transformation were the result of contamination of recipient cultures by small numbers of donor cells. This artifact is unlikely in the instance of DNA transfection because no donor cells could survive the deproteinization accompanying DNA preparation. In the case of chromatin transfection, inadvertant passage of viable cells together with chromatin was conceivable although still not likely in view of the 1% nonionic detergent used during chromosome preparation. Therefore, we wished to control the genetic origin of the donor and recipient cell lines. In the experiments described here, the donor cells were of C3H/He and BALB/c origin whereas the recipients were of NIH3T3 origin.

Southern blot analysis of cellular DNAs of these cell lines reveals a spectrum of proviruses of endogenous murine type C retroviruses (14). When *Eco*RI-cleaved cell DNA is probed with AKR virus cDNA, a characteristic and unique pattern of fragments is detected for each of the above-mentioned cell lines (unpublished results). We have used this spectrum as a characteristic signature of the genetic origin of a cell line under investigation. DNAs from the donor cell lines and from foci resulting from DNA and chromatin transfection were analyzed by this procedure to confirm their genetic origin. Prior to electrophoresis and Southern analysis, the DNA samples were encoded, and the identities were regenerated only after evaluation of the Southern blots. An example of this analysis is shown in Fig. 2. All transfected foci were found by this procedure to be NIH3T3 origin, whereas the donor cell lines were found to be of the expected C3H/He and BALB/c origin. This analysis precludes the potential donor cell contamination hypothesized above. However, this assay is not sensitive enough to determine how much of the donor DNA was established in the transfectant.

An Additional Transformed Phenotype of Transfectant Foci. The refractile foci induced among the recipient cells

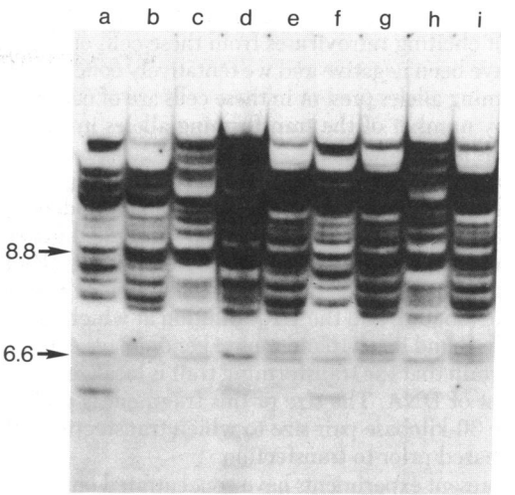

FIG. 2. Southern gel filter hybridizations of *Eco*RI-cleaved DNAs from different cell lines. DNAs of the three reference mouse strains and the derivative transformed and transfected cell lines were cleaved with endonuclease *Eco*RI and analyzed as described (12). The DNAs analyzed here are as follows. Lanes: a, C3H10T1/2; b, NIH3T3; c, BALB 3T3; d, MC5-5; e, focus derived from MC5-5 DNA; f, MCA5; g, focus derived from MCA5 DNA; h, DMBA-BALB 3T3; i, focus derived from DMBA-BALB 3T3 DNA. Numbers are in kilobases.

exhibit a distinct, readily distinguishable phenotype which is normally associated with transformation. To demonstrate transformation by a second criterion, transformed foci were isolated from monolayer cultures and tested together with their parental donor lines for their ability to form colonies in soft agar. Growth in this medium is usually associated with tumorigenicity and is widely used as a criterion of transformation (33, 34). As seen in Table 3, the normal NIH3T3 recipient cells did not form colonies, whereas most transformants and their respective parental donors grew to the 50- to 100-cell stage. Thus, by the criteria of morphology and anchorage independence, these transfected cells are transformed.

An exception to the transmitted anchorage independence was seen when examining the DMBA-BALB 3T3 cells used for chromatin transfection in which neither the donors nor the transfectants grew well in agar. Because neither the donor nor the transfected cells grew well in agar, this would not appear to represent an artifact of chromatin transfection. Rather it appears to reflect a weakly transforming phenotype present originally in this donor line, this phenotype being transmitted faithfully to the recipient cells upon chromosome transfection.

DISCUSSION

Studies of chemical carcinogenesis by Ames and others have suggested strongly that the carcinogenic event is a mutagenic event that alters the DNA of a target cell (1–5). Other work on *in vitro* carcinogenesis suggests that certain established cell lines can be transformed by carcinogens at rates consistent with one-hit kinetics (6, 7). These data suggested to us that a discrete, dominant allele may be present in certain chemically transformed cells whose introduction into a nontransformed counterpart would elicit transformation. The allele(s) studied here is capable of inducing transformation in NIH3T3 cells, an established cell line derived originally from outbred NIH/Swiss mice (21).

The high-efficiency donor cell lines studied here contain DNA which induces foci at the high efficiencies observed

previously upon transfection of retrovirus DNA (9). Our attempts at eliciting retroviruses from these cells or their derivatives have been negative and we tentatively conclude that the transforming alleles present in these cells are of cellular origin. The copy number of the transforming alleles in the DNA of these cells is unknown.

Although not directly demonstrated here, we consider it highly unlikely that the observed transformations depend upon successful introduction of two or more unlinked genetic elements into the same recipient cell. Given the low efficiencies of transfection [*ca.* 10^{-5} events per competent transfecting molecule (35, 36)] and the $1:10^6$ dilution at which single-copy genes are found in a haploid mouse genome, we consider it almost certain that the transforming trait is localized on a single fragment of DNA. The size of this fragment is probably less than the 30-kilobase pair size to which transfecting DNA has been sheared prior to transfection.

The present experiments have concentrated on the DNAs of high-efficiency donors whose transfection readily yields foci. These high-efficiency donors represent less than half of the transformed mouse lines that we have examined. The remaining low-efficiency donors may contain alleles that are transmissible via chromosome transfection. One such chromosome-mediated transmission was reported here. Interpretation of these chromosome transfection experiments is less clear because such transmissions do not prove that the allele is encoded solely by a discrete allele present in the DNA.

The ability to transmit the transformed phenotypes of five different cell lines suggests that these cells contain alleles that act dominantly in the NIH3T3 genetic background. These alleles may represent cellular genes whose alteration by the carcinogens resulted in their activation as transforming elements. The present studies show that two different carcinogens are able to induce these alleles in the DNAs of at least two strains of mouse cells. The transmission of these alleles via naked DNA would support the notion that DNA is the target of carcinogenesis and the carrier of the transformed trait. This transmissibility should make possible the isolation of the sequences encoding these alleles.

Note Added in Proof: Recent work by L. C. Padhy and R. A. Weinberg has shown that cells transformed by DNA transfection are tumorigenic in newborn mice.

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