Transfer of Sensitivity to Tumor Promoters by Transfection of DNA from Sensitive into Insensitive Mouse JB6 Epidermal Cells

NANCY H. COLBURN,^{1*} CATHERINE B. TALMADGE,³ AND THOMAS D. GINDHART²

Laboratory of Viral Carcinogenesis¹ and Laboratory of Experimental Pathology,² National Cancer Institute, and Program Resources Inc.,³ Frederick, Maryland 21701

Received 22 November 1982/Accepted 6 April 1983

Sensitivity to promotion of transformation by tumor promoters in mouse epidermal JB6 cells appears to have a genetic basis since the phenotypes of both promotable and nonpromotable JB6 cells derived from a common parent line are stable. Hybridization of promotable (P^+) and nonpromotable (P^-) cells previously indicated that promotability appears to behave as a dominant trait. These results suggest that it should be possible to find DNA sequences which specify sensitivity to promotion of anchorage independence by 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA). Cellular DNA isolated from one of two P^+ lines, JB6 Cl 41 or JB6 Cl 22, was CaPO_4 precipitated and used to transfect the P^- cell line JB6 Cl 30. At 7 days posttransfection, the cells were suspended in agar with or without TPA at 1.6×10^{-8} M and assayed 10 days later for TPA-dependent colony formation. Untreated or Cl 30 DNA-treated P^- JB6 Cl 30 cells yielded 40 to 50 colonies per 10^5 cells. In contrast, transfection of Cl 30 cells with " P^+ DNA" derived from either Cl 41 or Cl 22 yielded 200 to 500 TPA-induced colonies per 10^5 cells, or a five- to eightfold enhancement of promotability. The enhanced promotability obtained after transfection with P^+ DNA was stable, as judged by the retention of promotability for at least eight passages in cell lines derived from TPA-induced agar colonies. Other transfectants showed irreversible transformation by TPA, as observed in the parental P^+ lines. When NIH 3T3 cells instead of the putative preneoplastic JB6 Cl 30 cells were used as recipients for transfection of P^+ DNA, no evidence for acquisition of promotability was obtained. P^- JB6 Cl 25, like Cl 30, also permitted expression of transfected P^+ DNA. These results suggest that sensitivity to phorbol ester promotion of transformation in JB6 cells is determined by DNA sequence(s) present in the P^+ DNA and requires recipient cells of the appropriate phenotype for expression.

Whether there exists a genetic basis for susceptibility to tumor promoters is one of the important unanswered questions in carcinogenesis. Answering this question with *in vivo* initiation-promotion models (1, 3) has been difficult because tumor promotion is assumed to occur in a minor subpopulation consisting of carcinogen-initiated or post-initiated cells. Because such "promotable" subpopulations of carcinogen-exposed cells have not yet been identified in animals, one must study heterogeneous populations. For studying the genetics of tumor promoter sensitivity, clonal lines of initiated or post-initiated cells in culture can be useful. To be analogous to the mouse skin initiation-promotion model, initiated cells should have two essential features: (i) they must be stably nonneoplastic, and (ii) they must require exposure only to a tumor promoter, not to a complete carcinogen, to become irreversibly neoplastic. Post-

initiated cells, i.e., those which have undergone early-stage promotion (10, 24) should differ from initiated cells by requiring a relatively short period of exposure to tumor promoter. The mouse JB6 epidermal cell model which we have developed satisfies the criteria indicated above for post-initiated cells.

JB6 cells, originally established as a nonclonal line from untreated primary BALB/c mouse epidermal cultures, are nontumorigenic and anchorage dependent (7) and retain an epidermal specific cell surface antigen (2). Exposure of these cells to phorbol esters and other tumor promoters irreversibly promotes anchorage independence and tumorigenicity (4). Promotion of anchorage independence occurs when JB6 cells are exposed to second-stage (24, 25) but not first-stage tumor promoters and is inhibited by second-stage, not first-stage, promotion inhibitors (8). The JB6 model system thus appears to

be analogous to second-stage promotion as described by Slaga and co-workers (24, 25). Non-selective cloning of JB6 has yielded both promotion-sensitive and promotion-insensitive clones (5). Figure 1 illustrates the phenotypic characterization of promotable (P^+) and nonpromotable (P^-) JB6 cell lines. Cross-sensitivity to phorbol esters and other classes of tumor promoters was found in P^+ cells (5).

These sensitive and insensitive JB6 clonal cell lines were used in the studies reported here to determine whether sensitivity to promotion of transformation is specified by the DNA of promotable cells as demonstrated by DNA transfection. A genetic basis for sensitivity to promotion of transformation is suggested by our observation that both promotable (P^+) and nonpromotable (P^-) JB6 cell lines remain stable for many passages with respect to both anchorage independence and tumorigenicity phenotypes (8, 9). Assay of P^+/P^- hybrid cells reported elsewhere (6) suggests that promotability behaves as a dominant trait. Thus, the promotable phenotype might be expressed after a DNA-mediated gene transfer if the necessary determinants are encoded by P^+ DNA. Here we provide evidence that this is the case.

MATERIALS AND METHODS

Cells. Mouse JB6 cells were derived from untreated BALB/c primary epidermal cell cultures (7) and became sensitive to the induction of anchorage independence by 12-*o*-tetra-decanoyl-phorbol-13-acetate (TPA) at about passage 35 (4). Clonal lines of JB6 that were sensitive or resistant to promotion of transformation by tumor promoters were obtained from JB6 by nonselective cloning (5). NIH 3T3 cells were obtained from D. Blair, National Cancer Institute, Bethesda,

Md. JB6 cells were grown in Eagle minimal essential medium plus 8% fetal calf serum. NIH 3T3 cells were grown in Dulbecco minimal essential medium plus 10% calf serum.

Chemicals. TPA was obtained from Chemical Carcinogenesis, Eden Prairie, Minn. Stock solutions of 1 mg/ml in spectral-grade dimethyl sulfoxide were stored desiccated at -20°C . At the time of use, TPA was diluted to a final concentration of 10 ng/ml (1.6×10^{-8} M).

Assay for promotion of anchorage independence. Cells were suspended in 0.33% agar medium with or without the tumor promoter TPA and assayed for formation of colonies of ≥ 8 cells, scorable at 14 days as described previously (4). For assay of 10^5 cells, 100-mm dishes instead of 60-mm dishes were used. The anchorage-independent endpoint can also be assayed after TPA exposure in a monolayer (8).

DNA transfer by transfection. DNA was prepared by a 3-h proteinase K (100 $\mu\text{g/ml}$) treatment of sodium dodecyl sulfate cell lysates followed by extraction with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was ethanol precipitated, treated with 50 μg of RNase per ml for 2 h, then treated for 1 h with proteinase K, followed by phenol-chloroform-IAA treatment and dialysis. DNA transfection was carried out by the Parker and Stark (17) modification of the Graham and Vander Eb procedure (11). DNA at 150 to 200 $\mu\text{g/ml}$ was sheared through a 26-gauge needle and dissolved in 0.5 M CaCl_2 , and an equal volume of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) buffered saline, (pH 6.88) was added dropwise with agitation. After standing at room temperature for 30 min, 20 μg of the DNA-calcium phosphate precipitate suspended in 0.4 ml was added to a cell monolayer in a 60-mm dish containing 6×10^5 cells and 4 ml of 8% fetal bovine serum-containing medium. After 3 h at 37°C , the medium with DNA-calcium phosphate was removed, and the cells were treated with 15% glycerol in HEPES-buffered saline for 1 min. The medium was changed, and 1 day later the cells were subcultured 1:6. Promotability was assayed 1

FIG. 1. Promotion of transformation response in sensitive and resistant JB6 clonal lines.

TABLE 1. Promotability can be transferred by transfection of genomic DNA from P⁺ into P⁻ cells^a

DNA source	Pheno-type	Recipient cells	n	Promotion response (TPA-induced agar colonies per 10 ⁵ cells)
None		JB 6 Cl 30	6	40 ± 21
JB 6 Cl 30	P ⁻	JB 6 Cl 30	4	49 ± 41
JB 6 Cl 41	P ⁺	JB 6 Cl 30	7	225 ± 69
JB 6 Cl 22	P ⁺	JB 6 Cl 30	4	412 ± 105

^a Seven days after transfection of DNA (6 days after subculturing), the transfected JB 6 Cl 30 cells were suspended in agar ± TPA at 1.6×10^{-8} M. Results are given as mean number of TPA-induced agar colonies per 10⁵ cells ± standard deviation for the number of independent experiments indicated by n. P⁺, Promotable to anchorage independence and tumorigenicity by TPA and other tumor promoters, P⁻, nonpromotable.

week after transfection by suspending these cells in 0.33% agar ± TPA (10 ng/ml; 1.6×10^{-8} M).

Assay for stability of transfected phenotype. TPA-induced soft agar colonies produced by transfected cells were picked from agar (4) and grown as clonal cell lines in the absence of TPA. These clonal cell lines were assayed for colony formation in agar in response to TPA or to 0.1% dimethyl sulfoxide solvent only after the indicated number of passages.

RESULTS

The experimental approach used to determine whether DNA sequences in promotable cells carry information for sensitivity to promotion of anchorage independence by TPA involved transfection of DNA from P⁺ into P⁻ cells. The P⁻ recipient cells JB6 Cl 30 had previously been found to be efficient recipients for transfection of various retroviral DNAs (C. Talmadge and D. Blair, unpublished data). DNA was isolated from one of the JB6 P⁺ cell lines, sheared, calcium phosphate precipitated, and incubated with P⁻ cells, and the cells were glycerol treated as described above. The transfected cells were subcultured and assayed at 7 days for promotion of anchorage independence by TPA exposure in agar. Initial experiments indicated about the same number of colonies were formed in TPA-agar if the interval before TPA exposure ranged from 4 to 14 days. If the interval was 1 to 3 days, expression of the P⁺ phenotype did not occur (four of four experiments; data not shown). Also, no expression occurred without subculture with a 2-day interval before agar suspension.

Table 1 shows the results of transfecting cellular DNA from P⁻ or P⁺ cells into P⁻ Cl 30 cells. The yield of TPA-induced agar colonies was low after transfection of Cl 30 DNA and not significantly different from that obtained in the un-

transfected controls. After transfection of P⁺ DNAs from Cl 41 or Cl 22, the promotion response increased by a factor of five- to eight-fold over the P⁻ DNA background. Thus, DNA from promotable cells appears to have sequences that can confer promotion sensitivity on the promotion-resistant JB6 Cl 30 cell variant.

To determine the specificity of the Cl 30 P⁻ cells for permitting expression of the P⁺ phenotype after transfection of P⁺ DNA, JB6 Cl 25 P⁻ cells and NIH 3T3 cells were alternatively used as recipients. Table 2 shows a comparison of Cl 25 and NIH 3T3 cells with JB6 Cl 30 cells as recipients for transfection of P⁺ DNA. The P⁺ phenotype was not expressed in NIH 3T3 cells after P⁺ transfection, in contrast to parallel assays of the same DNAs transfected into Cl 25 or Cl 30 cells. Separate experiments employing cotransfection with a dominant selectable marker (*Escherichia coli* gpt gene [15]) supplied by G. Crouse and M. Pearson of the National Cancer Institute-Frederick Cancer Research Center) indicated that the lack of P⁺ expression after transfection into NIH 3T3 cells could not be explained by a lack of DNA uptake. Thus, expression of the P⁺ phenotype apparently depends on both donor DNA and recipient cells, with either of two P⁻ JB6 clonal lines showing the capacity to express transfected P⁺ DNA.

Since instability of various transfected phenotypes (13, 20, 28) has been reported, we investigated the stability of the P⁺ phenotype after transfection. After transfection of P⁺ DNA into JB6 Cl 30 cells, these cells were induced by TPA to form soft agar colonies. These TPA-induced colonies were plucked, established in the absence of TPA as clonal lines (with each clonal line indicated by a letter preceded by either 41 or 22, designating the P⁺ DNA transfected), and assayed for promotability for several passages (Fig. 2). Although one of these lines (22c, Fig.

TABLE 2. Expression of P⁺ phenotype after transfection depends on both donor DNA and recipient cells^a

Donor DNA	Recipient cells	n	Promotion response (TPA-induced agar colonies per 10 ⁵ cells)
JB 6 Cl 41	JB 6 Cl 30	4	444 ± 250
JB 6 Cl 41	JB 6 Cl 25	3	393 ± 102
JB 6 Cl 41	NIH 3T3	4	10 ± 17
JB 6 Cl 22	JB 6 Cl 30	4	478 ± 164
JB 6 Cl 22	JB 6 Cl 25	3	356 ± 75
JB 6 Cl 22	NIH 3T3	4	3 ± 6

^a Methods for transfection of P⁺ DNAs and for determination of promotion response are given in the legend to Table 1.

FIG. 2. (a) Retention of promotability transferred by DNA transfection. P^+ JB6 Cl 41 or Cl 22 DNA was transfected into P^- JB6 Cl 30 cells and suspended in agar $\pm$ TPA (1.8×10^{-8} M). TPA-induced colonies were plucked to establish the clonal lines indicated by letters. For each clonal line, the promotion of transformation response to TPA is expressed as the agar colony yield per 10^4 cells in TPA-treated samples minus the colony yield in untreated controls. This background level was usually <10 colonies per 10^4 cells for these lines. Psg Nbr, Passage number after cloning from agar plus TPA; 41, TPA-induced agar colony yield for the original transfectant obtained on transfection of Cl 41 DNA into Cl 30 cells; 22, TPA-induced agar colony yield for the original transfectant obtained on transfection of Cl 22 DNA into Cl 30 cells. (b) Irreversible transformation of a P^+ transfectant. After transfection of clone 22 DNA into Cl 30 cells as described in (a), a TPA-induced agar colony was established as a clonal line (22C) in the absence of TPA (along with 22A and 22B shown in [a]) and assayed for colony formation in agar $\pm$ TPA. Shown are the colony yields obtained for 22C with or without exposure to TPA versus passage number after cloning from agar plus TPA. The broken lines indicate the TPA-induced agar colony yield for the original transfectant obtained on transfection of Cl 22 DNA into Cl 30 cells.

2b) behaved like the parental P^+ lines (4) and showed stable, irreversible transformation after promotion of anchorage independence, the remaining transfected, cloned lines assayed (Fig. 2a) showed low to zero agar colony yields in the absence of TPA. When promotability was measured at the second to fourth passage after cloning from agar plus TPA, the values were significantly higher than the initial values, presumably reflecting a selection achieved by cloning from agar. Figure 2a shows that high (although variable) promotability values persisted until at least the seventh postcloning passage, indicating retention of the transfected P^+ phenotype. Subsequent studies have shown that, on the average, about 50% of the cell lines cloned from agar colonies after induction of P^+ transfectants by TPA are irreversibly transformed as in Fig. 2b. The other 50% show a P^+ phenotype as in Fig. 2a. This contrasts with a 90% frequency of irreversible transformants obtained from parental P^+ cells.

DISCUSSION

We conclude from these results that DNA from promotable cells must have sequences that

can confer promotion sensitivity on promotion-insensitive JB6 variants. Whether these sequences function to code for a missing or inactive gene product, as suggested by the apparent dominance of the P^+ phenotype in cell hybrids (6), is an important question to be investigated. If the P^+ cells contain DNA sequences which code for a protein that is inactive or missing from the P^- cells, the question arises as to what such a protein might be. Recent studies in our laboratory have elucidated a biochemical response to TPA that consistently occurs in promotion-sensitive but not promotion-resistant cell lines, namely, a 90% decrease in the synthesis of cell surface trisialoganglioside (9, 26, 27). Thus, one might postulate that the P^+ specific gene product is a TPA-induced neuraminidase which catalyzes the hydrolysis of trisialogangliosides. Support for this possibility comes from initial experiments with a cloned P^+ transfectant line which indicate that the trisialoganglioside response to TPA is transferred along with promotability into a P^- cell line (L. Srinivas, C. Talmadge, and N. Colburn, unpublished data).

Another possibility is that the P^+ gene is a transforming gene such as those which can

transfer the tumor cell phenotype on transfection, as described by a number of investigators (12, 14, 16, 18, 19, 21–23). In this case, the expression of this gene may be regulated by TPA in the P⁺ cells and not in the P⁻ recipients, but may be expressed independently of TPA in the transformed cells.

Current efforts are aimed at characterizing and cloning the DNA sequences responsible for the P⁺ phenotype. Initial experiments indicate that the Cl 22 P⁺ activity is sensitive to a number of restriction endonucleases, including *EcoRI*, *BamHI*, *HindIII*, *PvuI*, *PstI*, and *PvuII*, but not to *BglI* and *BglII* (M. Cohen, N. Colburn, C. Talmadge, and G. Hegamyer, unpublished data). In addition, DNA from a P⁺ transfectant obtained after transferring Cl 22 DNA showed the same restriction enzyme sensitivity on secondary transfection.

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