Transfer by pro Gene Transfection of Tumor Promoter-sensitive Phenotype to Promotion-insensitive JB6 Cells


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ABSTRACT

Transfection of activated promotion sensitivity genes (pro genes) confers on insensitive (P−) cells susceptibility to induction of anchorage-independent growth by tumor-promoting phorbol esters. Promotion-sensitive (P+) JB6 cell variants, from which activated pro-1 and pro-2 were cloned, respond to 12-O-tetradecanoylphorbol-13-acetate (TPA) and various nonphorbol tumor promoters with anchorage-independent transformation that is irreversible 60–80% of the time. Anchorage-independent (Tx) clonal lines derived from these TPA-induced agar colonies were also tumorigenic in nude mice. This report has addressed the question of whether the phenotypes associated with parental P+ cells are transfected by transfection of activated pro-1 and pro-2. Clonal lines were established after transfection of JB6 P− cells with activated pro-1 or pro-2, induction of anchorage-independent colony formation by TPA, and growth of individual agar colonies to yield clonal transflectant lines. The lines so derived from transfected populations included Tx, P+, and P− lines, reflecting irreversible neoplastic transformation and greater and lesser degrees of preneoplastic progression, respectively. The anchorage-independent transflectants were found to be tumorigenic. Since untransfected P− cells subjected to the same single-cycle TPA treatment and cloning in agar yielded no anchorage-independent and few P+ transflectants, the appearance of P+ and Tx transflectants after pro-1 and pro-2 transfection is therefore likely to be due to the transfected pro genes. Indirect assay of pro gene uptake by quick-blot hybridization of transflectant cell DNA with the vectors into which pro genes had been cloned confirmed the association of transferred P+ and Tx phenotypes with the presence of the transfected DNA. Finally, assay of the sensitivity of P+ pro-1 and pro-2 transflectants to transformation by TPA at various concentrations showed that transfaction with pro-1 or pro-2 conferred about equal responses that were somewhat lower than those observed with parental P+ controls. Taken together these data indicate that promotion-insensitive JB6 cells need only an activated pro gene and TPA exposure to become neoplastically transformed.

INTRODUCTION

Evidence for genetic determinants of susceptibility to tumor promotion has come from the observation that mice can be bred for increased or decreased sensitivity to skin tumor promotion without coselection for parameters associated with tumor initiation (1–4). It can also be said that the process of initiation, achievable by H-ras activation (5), renders mice susceptible to tumor promotion. It is clear, however, that the genetic loci involved in breeding for promotion sensitivity do not render promotion-sensitive mice initiated. That is, these mice need to be initiated (and subsequently promoted) to show significant tumor yields. Hence, it follows that the genetic loci involved in initiation and those conferring promotion sensitivity in promotion-sensitive mice are separate loci.

The mouse JB6 epidermis-derived variant cell lines (6, 7) are being used as a model system to investigate what may be the genes that confer promotion sensitivity in the mice described above. These cell lines are spontaneously immortalized, spontaneously initiated lines that differ in sensitivity to tumor promoter-induced neoplastic transformation. JB6 P− cell variants are sensitive to promotion of neoplastic transformation by TPA+ and other tumor promoters, as shown by tumor promoter-induced development of anchorage independence and tumorigenicity (6, 7). JB6 P+ cell variants are insensitive to promotion of transformation by TPA+ and other tumor promoters (6, 7). The yield of TPA-induced anchorage-independent colonies in P− cells is typically less than 1% of the value obtained with P+ cells, and the P− colonies are smaller. Transfer of promotion sensitivity to P− cells can be achieved by transfection with P+ but not P− DNA (8, 9). Recently two promotion sensitivity genes, termed pro-1 and pro-2, have been cloned from JB6 P+ cell DNA (10). Either pro-1 or pro-2 transfers to P− cells sensitivity to TPA-induced anchorage-independent transformation. Although the nucleotide sequences of pro-1 and pro-2 are completely different from each other and from other known genes, these sequences are similar to each other in at least two respects. (a) Either pro-1 or pro-2 can transfer with similar molar specific activity to human genetically cancer-prone BCNS fibroblasts a substantial extension of lifespan (11). This pro gene-induced "escape from senescence" was seen when BCNS but not normal human fibroblasts were the recipients, even though the transfected DNA was stably retained for several passages in the latter (11). This suggests that activated pro genes can collaborate with BCNS genes to produce an extended lifespan. In the mouse JB6 system pro genes can apparently cooperate with genes expressed in P+ cells (but not with genes expressed in NIH 3T3 cells (8)) to produce promotion sensitivity.

The present report addresses the issue of whether these phenotypes associated with parental P+ cells are stably transfected with activated pro genes. We have asked whether the degree of sensitivity to TPA shown by P+ transflectants is similar to that for parental P+ cells. We have also asked whether a subset of the TPA-transformed anchorage-independent clones derived from P+ transflectant cells resemble parental cells in being irreversibly transformed to neoplastic phenotype. That is, in order to be capable of conversion into tumor cells, do the P− cells need only acquisition of an activated pro gene and tumor promoter exposure?

MATERIALS AND METHODS

A Novel Neoplastic Phenotype is Induced by Transfection of Parental P+ JB6 Cells

Clonal lines of JB6 P− cells were transfected with pro-1 or pro-2 plasmids. The pro-1 containing plasmid p26 contained a P− plasmid. The pro-1-containing plasmid p26 contained a P− plasmid.
controls are shown in Fig. 2. Of 22 clones, none had undergone passages 6-19 for clones 3-22. The phenotypes were stable assayed 3-4 times at passages 5 to 20 for clones 1 and 2 and at The TPA-induced colony size was smaller for P than for P@ produced agar colonies. These untransfected P recipient cells were produced as described previously (8, 10). The typical yield of P@ DNA-dependent colonies was 100-150 per 10^6 cells while of P DNA-independent (P DNA or no DNA) colony yield was carried out. This assay was also used to generate cloned pro-1 or pro-2 transfectants from TPA-induced agar colonies.

Assay for P+, P-, and Tx Phenotypes. Cells were suspended in 0.33% agar containing 10% fetal calf serum with or without TPA at indicated concentrations (6). P- phenotype is characterized by less than 200 TPA-induced and less than 10 non-TPA-induced colonies per 10^6 cells, P+ by greater than 200 TPA-induced colonies per 10^6 cells, and Tx by greater than 200 anchorage-independent colonies in the absence of TPA.

Assay for Gene Transfer by Quick-Blot Hybridization. The procedure was based on those originally described by Bresser et al. (16) and further modified by Porteous (17). Briefly, 10^6 cells were lysed in 0.1 ml of 0.5% sodium dodecyl sulfate, 100 mM Tris Cl and 100 mM EDTA, pH 7.5, digested with proteinase K (100 μg/ml) for 2 h at room temperature, and then sonicated for 10 sec. Finally, cell lysates were extracted twice with phenol, then phenol-chloroform-isooamyl alcohol (25:24:1). To 0.1 ml of cell extract (equivalent of 10^6 cells) 81.3 μl of supersaturated NaI (2.5 g/ml in hot water) were added, bringing the solution to 12.2 molal with respect to NaI. After heating at 90°C for 10 min, samples were rapidly applied under suction to S&S BAB5 nitrocellulose on a MiniFold apparatus (Schleicher & Schuell). The filters were washed twice with 70% ethanol and once with 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine. Nitrocellulose filters were hybridized with oligolabeled pCD-X plasmid DNA at 68°C for 24 h in 0.1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate, 5X standard saline citrate, 5× Denhardt's solution, and 5% dextran sulfate. Filters were washed 4 times at 52°C to 2× standard saline citrate and radioautographed at -70°C using DuPont Cronex film with intensifier screens.

RESULTS

JB6 P- cells were transfected with cloned pro-1 or pro-2 (10). The plasmids used were p26, containing the entire 1.05-kilobase pair sequence of pro-1 necessary for biological activity and pCC38 containing the 3.8-kilobase biologically active sequence of pro-2 (see Fig. 1). After suspension in agar at 4 days post-transfection, TPA-induced anchorage-independent colonies were produced as described previously (8, 10). The typical yield of P+ DNA-dependent colonies was 100-150 per 10^6 cells while the P- DNA-dependent (P- DNA or no DNA) colony yield was 30-50 per 10^6 cells (10). Transfected populations of pro-1 and pro-2 gave rise to clonal lines established from individual TPA-induced agar colonies (Fig. 1). In parallel, nontransfected recipient cells were established as clonal lines from TPA-induced agar colonies. These untransfected P- recipient cells showed a low frequency of agar colony induction by TPA in the range of 0.1 to 1% of the values seen with P+ cells (6, 18, 19). The TPA-induced colony size was smaller for P- than for P+ cells.

The phenotypes (P+, P-) of the nontransfected TPA-induced controls are shown in Fig. 2. Of 22 clones, none had undergone irreversible anchorage-independent transformation. Two of the 22 became P+ and 20 remained P- . The phenotypes were assayed 3-4 times at passages 5 to 20 for clones 1 and 2 and at passages 6 to 19 for clones 3 to 22. The phenotypes were stable for at least the range of passages indicated. Clonal lines established from pro-1 or pro-2 transfected populations were assayed for acquisition of P+ or Tx phenotype. The pro-1 transfectants (Fig. 3) showed 1 of 8 (clone 6) had undergone anchorage-independent transformation. Three (clones 4, 9, and 11) became P+ and 4 (clones 1, 3, 5 and 12) remained P-. These phenotypes were assayed 3-5 times between passages 6 and 23 to yield the means shown. Of the pro-2 transfectors (Fig. 4) 2 of 20 (clones 16 and 18) were anchorage-independently transformed, 5 (clones 7, 8, 10, 11, and 15) showed P+ phenotype and 13 (clones 1, 2, 3, 5, 6, 9, 12, 13, 17, 19, 23, 24, and 25) showed P- phenotype. These phenotypes were assayed 3-5 times between passages 2 and 24 to yield the means shown. Thus, of 8 clonal lines established from pro-1 transfected populations 50% were irreversibly P+ or Tx and of 20 lines from pro-2 transfected populations 35% were P+ or Tx. This is to be contrasted with 9% P+ of 22 clones from untransfected populations. The observed progression was in all cases irreversible, i.e., no P+ or Tx clones reverted to P- phenotype.

If manifestation of P+ phenotype requires only the acquisition of an active pro gene, then those cell lines cloned from transfected TPA-treated populations that stably integrated and ex-
pressed activated pro genes should show P' or Tx phenotype. Those that did not stably integrate or that integrated but did not express activated pro genes would be expected to remain P-. Fig. 5 shows a quick blot analysis of the presence of sequences hybridizable to pCD-X, the vector into which pro-1 had been cloned in transfectant cell DNA. A mouse pro-1 probe could not be utilized since it would hybridize to pro-1 homologous sequences present in P- DNA. Vector hybridizable sequences were found in P26 clones 4, 11, and 6 that showed P', P+, and Tx phenotypes, respectively (Fig. 5). Lack of hybridization (i.e., signal indistinguishable from untransfected Cl 30 cells) was seen with the DNA of P transfectants clones i2 and 12. Thus 7 of the 7 vector-negative clones assayed showed P' phenotype, while 9 of 9 P+ or Tx transfectants were vector positive.

JB6 P+ cells when transformed to anchorage independence by TPA characteristically show tumorigenicity as well (20, 21). Also found to be tumorigenic in nude mice were pro-1 transfectant Cl 6 and pro-2 transfectants Cl 16 and 18, which had been transformed by TPA treatment in agar to anchorage independence and were vector positive (Table 1). Similarly cloned transfectants that showed P+ phenotype were, like P+ parental cells, not tumorigenic (Table 1), nor were P- transfectants tumorigenic (not shown). This suggests that in order to be capable of conversion to tumorigenic phenotype, P- cells need only acquisition of an activated pro gene and TPA exposure.

Although Figs. 3 and 4 established that populations of P+ cells subjected to transfection with cloned pro-1 or pro-2 gave rise to subpopulations of cells susceptible to TPA-induced anchorage independence, these data did not establish the degree of TPA sensitivity of these P+ transfectants. Fig. 6 shows a comparison of pro-1 and pro-2 P+ transfectants with parental P+ Cl 41 cells for sensitivity to TPA-induced anchorage independence. At 10 ng/ml TPA in the top agar layer, pro-1 transfectants showed responses similar to parental P+ cells while pro-2 transfectants were less responsive. One-tenth the concentration showed 4 of 5 transfectants to have reduced sensitivity. At lower concentrations pro-1 transfectants showed TPA sensitivity similar to that for pro-2 transfectants.

DISCUSSION

These results have shown that pro-1 and pro-2 transfectant cell lines established from TPA-induced agar colonies after calcium phosphate transfection of P- recipient cells number among them clones that show P-, P+, or Tx phenotypes. The strategy used for producing clonal transfectants did not utilize a dominant selectable marker that would permit elimination of cells that failed to take up DNA but instead utilized selection based on susceptibility to TPA-induced anchorage independence. Since untransfected P- cells show a low but detectable
Table 1  Tumorigenicity of pro-1 and pro-2 transfectants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Transfectant</th>
<th>Phenotype in anchoragin-</th>
<th>Transfected with</th>
<th>Selected in ±TPA (no. of cycles)</th>
<th>Tumorigenicity in nude mice (no. of positive/total alive)</th>
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<tbody>
<tr>
<td>Controls</td>
<td></td>
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<tr>
<td>JB6 CI 30 (13)</td>
<td>JB6 (12) (unclosed)</td>
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<td>-</td>
<td>-</td>
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<td>JB6</td>
<td>P*</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>RT 101 (13)</td>
<td>JB6 CI 41</td>
<td>T-</td>
<td>-</td>
<td>-</td>
<td>+ (6)</td>
<td>10/12</td>
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<tr>
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<td>JB6</td>
<td>T-</td>
<td>-</td>
<td>+</td>
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<tr>
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<tr>
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<td>-</td>
<td>+</td>
<td>+ (1)</td>
<td>0/8</td>
</tr>
<tr>
<td>41 T CI 9 (14)</td>
<td>JB6 CI 41</td>
<td>T-</td>
<td>-</td>
<td>+</td>
<td>+ (1)</td>
<td>4/21</td>
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<tr>
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<td>JB6 CI 41</td>
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<td>JB6 CI 30</td>
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<td>p26</td>
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<td>p26 CI 6</td>
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<td>p26</td>
<td>+</td>
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<td>JB6 CI 30</td>
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<td>2/8</td>
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<td>+</td>
<td>+ (1)</td>
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anchor independence response to TPA (Fig. 2), it was anticipated that TPA selection (and/or induction) of \( \text{pro} \) gene transfected populations would yield some "background" clonal lines that showed \( \text{P}^- \) phenotype. If integration and expression of a \( \text{pro} \) gene causes acquisition of \( \text{P}^+ \) or \( \text{Tx} \) phenotype, then one would predict that the frequency of \( \text{P}^+ \) or \( \text{Tx} \) clonal lines should reflect the ratio of \( \text{P}^+ \) DNA-dependent to \( \text{P}^- \) DNA-independent TPA-induced colony yield, a ratio of 2 to 5 (see "Results" and Fig. 1). Thus 50 to 80% of the clones established should be \( \text{P}^- \) or \( \text{Tx} \). This is likely, however, to be an overestimate since it assumes that all colonies that initially harbor and express activated \( \text{pro} \) sequences retain them in subsequent passages. The 50% of \( \text{P}^- \) or \( \text{Tx} \) clones from \( \text{pro}-1 \) transfected populations and 35% of \( \text{P}^- \) or \( \text{Tx} \) clones from \( \text{pro}-2 \) populations therefore appear close to that predicted by the hypothesis.

The hypothesis that stable integration and expression of activated \( \text{pro} \) gene causes acquisition of \( \text{P}^+ \) or \( \text{Tx} \) phenotype also predicts that \( \text{P}^- \) and \( \text{Tx} \) transfectants should harbor vector sequences with a frequency approaching 100%. These results showed that 100% of nine tested \( \text{P}^- \) or \( \text{Tx} \) clonal lines were vector positive. Another prediction of the hypothesis is that of the clonal lines found to be vector negative, these should be \( \text{Tx} \) with a 0% frequency and \( \text{P}^+ \) with a frequency approaching 0% (see Fig. 2). [Actually the expected frequency of \( \text{pro} \) gene-independent \( \text{P}^+ \) clones should be 9% (2 of 22) of 20–50% or about 2–5%.] In fact the results showed that 0% of the seven vector negative clones were \( \text{P}^- \) or \( \text{Tx} \). The \( \text{pro}-2 \) clonal line (clone 12) that showed some vector hybridization may have harbored \( \text{pro}-2 \) that was incomplete or was integrated in a manner inappropriate for expression. Thus the data show co-selection for vector sequences with \( \text{P}^- \) or \( \text{Tx} \) phenotype and support the possibility that acquisition of \( \text{P}^+ \) or \( \text{Tx} \) phenotype was due to acquisition of active \( \text{pro} \) gene. This interpretation should, however, be a cautious one in view of the fact that the assay used to track the presence of \( \text{pro} \) sequences was an indirect one. Current studies are focused on developing a direct assay.

Of the four \( \text{pro}-1 \) transfectants exhibiting a \( \text{P}^+ \) or a \( \text{Tx} \) phenotype (Fig. 3), one (25%) was irreversibly transformed after one cycle through agar + TPA. For \( \text{pro}-2 \) transfectants the corresponding number irreversibly transformed was two of seven (29%) (see Fig. 4). The frequency with which Colburn et al. have observed irreversible transformation of parental JB6 \( \text{P}^- \) cells after one cycle of TPA induction in agar has been approximately 70% (6, 14). On the other hand, Takahashi et al. (21) observed a frequency of less than 10% on the first cycle but higher frequencies of irreversible anchorage-independent and tumorigenic transformation on the second through fifth cycles of TPA induction in agar. Hence the 25 and 29% frequency of irreversible transformation after one cycle of TPA exposure in agar is in the range of reported values for TPA-exposed parental JB6 \( \text{P}^- \) cells.

The transfectants that were stably \( \text{P}^+ \) (seen in Figs. 3 and 4) can shed light on the nature of the phenotypes transferred by activated \( \text{pro}-1 \) or \( \text{pro}-2 \). The sensitivity to TPA-induced transformation was about equal for \( \text{pro}-1 \) and \( \text{pro}-2 \) transfectants but both were somewhat less sensitive than parental \( \text{P}^+ \) cells at higher TPA concentrations. These data do not distinguish the phenotype specified by \( \text{pro}-1 \) from that specified by \( \text{pro}-2 \). The parental \( \text{P}^- \) phenotype is characterized by sensitivity to promotion of transformation by several nonphorbol tumor promoters as well as by sensitivity to antipromotion by several inhibitors. A report being submitted separately considers the sensitivity of these clonal transfectants to several nonphorbol tumor promoters and to several antipromoters and describes two salient characteristics that distinguish \( \text{pro}-1 \) and \( \text{pro}-2 \) transfectants from each other or from parental \( \text{P}^+ \) cells.²

All five of the anchorage-independent transformants tested were vector positive and tumorigenic; no vector-negative (all \( \text{P}^- \) lines tested became tumorigenic. This indicates that in order for preneoplastic JB6 \( \text{P}^- \) cells to be converted into neoplastic cells they need only acquire an activated \( \text{pro}-1 \) or \( \text{pro}-2 \) and be exposed to TPA. Unlike certain other immortalized mouse cells [e.g., NIH 3T3 cells (8, 10)], \( \text{P}^- \) cells are permissive for expression of the biological activity of activated \( \text{pro}-1 \) or \( \text{pro}-2 \) when transfected. This suggests that \( \text{P}^- \) cells harbor a collaborating gene or lack a dominant \( \text{pro} \) gene suppressor that may exist in nonpermissive recipients. Barrett and coworkers (22) have presented evidence that later stages of preneoplastic progression in Syrian hamster cell transformation involve loss of a suppressor function. Such a loss of suppressor function might have occurred during the progression that led to the \( \text{P}^- \) phenotype in JB6 cells.

The DNA of TPA-transformed \( \text{P}^- \) parental cells has been found to carry a transforming activity separate from the \( \text{P}^+ \) activity (23). We postulate that activated \( \text{pro}-1 \) or \( \text{pro}-2 \) with TPA can function to "switch on" a separate transforming gene which then acts to maintain the tumor cell phenotype.

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² Colburn et al., unpublished data.
REFERENCES


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