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Genes That Cooperate With Tumor Promoters in Transformation

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Tumor-promoting phorbol esters, like growth factors, elicit pleiotropic responses involving biochemical pathways that lead to different biological responses. Genetic variant cell lines that are resistant to mitogenic, differentiation, or transformation responses to tumor promoters have been valuable tools for understanding the molecular bases of these responses. Studies using the mouse epidermal JB6 cell lines that are sensitive or resistant to tumor promoter-induced transformation have yielded new understanding of genetic and signal transduction events involved in neoplastic transformation. The isolation and characterization of cloned mouse promotion sensitivity genes *pro*-1 and *pro*-2 is reviewed. A new activity of *pro*-1 has been identified: when transfected into human cancer prone basal cell nevus syndrome fibroblasts but not normal fibroblasts mouse *pro*-1 confers lifespan extension on these cells. Recently, we have found that a *pro*-1 homolog from a library of nasopharyngeal carcinoma, but not the homolog from a normal human library, is activated for transferring promotion sensitivity. The many genetic variants for responses to tumor promoters have also proved valuable for signal transduction studies. JB6 P− cells fail to show the 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced synthesis of two proteins of 15 and 16 kD seen in P^+ cells. P^-, P^+, and TPA transformed cells show a progressive decrease in both basal and TPA-inducible levels of a protein kinase C substrate of 80 kD. P^- cells are relatively resistant both to anchorage-independent transformation and to a protein band shift induced by the calcium analog lanthanum. It appears that one or more calcium-binding proteins and one or more *pro* genes may be critical determinants of tumor promoter-induced neoplastic transformation.

Key words: genes specifying sensitivity, tumor promotion, neoplastic transformation

Tumor-promoting phorbol esters, like growth factors, elicit pleiotropic responses. Some 40 biochemical or biological responses to tumor promoters have been described [1]. Emerging findings in several laboratories (see Table I) [refs. 2–38] suggest that subsets of tumor promoter-elicited responses may lead to mitogenesis, differentiation, or neoplastic transformation. The many genetic variants now available (Table I) can be valuable tools for assigning a response to one of these pathways. One

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TABLE I. Genetic Variants for Responses to Tumor Promoters

Cells or tissue	Variant parameter	References
HL-60, human promyelocytic leukemia	Phorbol diester receptor down modulation	[2]
El-4 mouse thymoma	Protein kinase C substrates	[3]
Balb/3T3 mouse	$K^+/Na^+/Cl^-$ transport	[4]
Caenorhabditis elegans	Shrinking movement responses	[5,6]
Initiation-promotion sensitive mouse strains: epidermis	Sustained hyperplasia	[7]
Nude mouse epidermis	Hyperplasia	[8]
Cultured mouse mammary glands: Mtv-2^+	DNA synthesis stimulation	[9]
Human FP fibroblasts	Mitogenic response	[10]
Swiss 3T3 mouse	Mitogenic response	[11–14]
Swiss 3T3 mouse	Gene amplification	[15]
J6B mouse epidermal	Mitogenic response	[16,17]
EL-4 thymoma	Interleukin-2 induction	[3]
HL-60	Differentiation responses	[18–20]
FELC	Differentiation responses	[21]
LC mouse kerantinocyte cell lines	Terminal differentiation	[22–24]
Human keratinocytes normal; transformed	Terminal differentiation	[25]
Human bronchial epithelial cells; normal; tumor	Terminal differentiation	[26]
Human ataxia telangectasia fibroblasts	Cytotoxicity response	[27]
Human lymphoma cells: EBV^+; EBV^-	Cytotoxicity response	[28]
Ad-5 rat embryo fibroblasts	Transformation progression response	[29,30]
Rat fibroblasts + PyLT or *myc*	Transformation promotion response	[31]
10 T 1/2 mouse embryo fibroblasts + T24	Transformation promotion response	[32]
Rat embryo fibroblasts + activated *ras*	Transformation promotion response	[33]
JB6 mouse epidermal	Transformation promotion response	[34–37]
HSV infected NIH 3T3 cells	Promotion response	[38]

can deduce, for example (Table I), that sensitivity to promotion of neoplastic transformation by phorbol esters can be specified by an activated *ras* [32,33] or by activated *myc* or polyoma large T [31] or by activated *pro* genes [37]. Just how it is that any of these genes cooperates with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to produce neoplastic transformation is not yet clear. The focus of this chapter is to describe what has been learned about promotion-relevant genes and signal transduction in studies using mouse JB6 cells.

As shown in Figure 1, the mouse epidermal JB6 model system shows several of the characteristics of second-stage tumor promotion in mouse skin [39–41]. Incomplete second-stage tumor promoters such as mezerein or retinoyl phorbol acetate (as

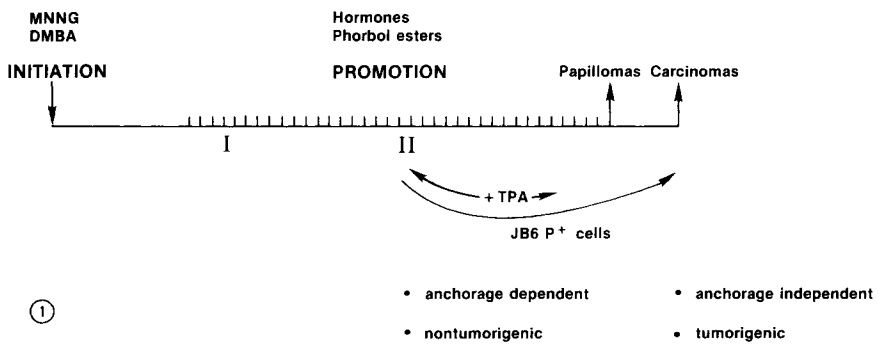

Fig. 1. The JB6 model system: an analog of second-stage mouse skin tumor promotion.

well as complete tumor promoters) induce the transition from nontumorigenic to tumorigenic phenotype. The process is blocked by inhibitors of second-stage tumor promotion such as retinoids but not by first-stage inhibitors such as antiproteases. The period during which the in vivo progression to carcinomas is dependent on continued exposure to tumor promoter ends with the benign papillomas [42], a subpopulation of which are precarcinomatous [43] and require only expression time to give rise to squamous carcinomas. Similarly, tumor-promoter exposure of JB6 promotion-sensitive (P^+) cells can be stopped prior to the appearance of soft agar colonies and coincident tumorigenicity without reduced yield of transformants. This indicates that the benign-to-malignant transition has been irreversibly set in motion by tumor-promoter exposure both in vivo and in JB6 cells at a stage that is premalignant.

GENES THAT SPECIFY PROMOTION SENSITIVITY: *PRO*-1 AND *PRO*-2

Two new putative genes that specify sensitivity to promotion of neoplastic transformation have been cloned by sib selection from a genomic library of JB6 P^+ cells [37]. These sequences, designated *pro*-1 and *pro*-2, are different from each other and from known oncogenes.

Sib selection or successive subdivision of active pools first introduced by Cavalli-Sforza and Lederberg [44] offers an approach to isolating genes for which only a biological activity assay is available (ie, when specific molecular probes or antibodies are not available for screening a library). When the DNA from a genomic library is transfected and is shown to transfer the biological activity associated with genomic DNA from the parental cells, a sib selection applied to that library offers a straightforward unbiased way to retrieve and purify DNA sequences that can transfer the activity in question.

In the case of the *pro* genes, five cycles of sib selection identified 20–30% of the pools at each cycle as active [37]. Choosing the single most active pool at each cycle for further analysis finally yielded two active *pro* sequences. It is expected that other active pools not analyzed would yield additional *pro* sequences, as well as reisolates of *pro*-1 and *pro*-2. *Pro*-1 and *pro*-2 can each transfer promotion sensitivity to resistant JB6 P^- cells with a similar specific activity [37]. The transfer of P^+ activity saturates at about 10^{-17} mol *pro* gene (about 200 pg plasmic DNA) per

transfection dish (6×10^5 cells), yielding a maximal number of about 100 TPA-induced transfected-DNA-dependent colonies per 10^5 cells. Dose dependency of P^+ activity transferred occurs in the range of about 10^{-18} to 10^{-17} mol *pro* gene per transfection dish [37].

Table II shows a comparison of the characteristics of cloned mouse *pro*-1 and *pro*-2. *Pro*-1 is intronless with an open reading frame of a size that could specify a 7,000-dalton protein or an RNA of 1,000 nucleotides or less. *Pro*-1-hybridizable RNA levels are increased by exposure of JB6 cells to TPA. The stimulation is greater in P^+ than in P^- cells [45]. Although mouse *pro*-1 is composed of sequences complementary to mouse repeats BAM 5 and B1, *pro*-1 appears to occur at a low copy number in the human genome [37,46]. *Pro*-2 appears to be a single-copy gene in both the mouse and the human genomes and to be expressed in mouse skin carcinomas and papillomas [37,46].

ACTIVITIES OTHER THAN PROMOTION SENSITIVITY THAT ARE SPECIFIED BY *PRO* GENES: LIFESPAN EXTENSION IN HUMAN BCNS CELLS

A number of genes including transforming genes have been found to confer upon transfection different activities depending on the recipient cells used. *Myc*, for example, can confer immortalization or can collaborate with activated *ras* in transformation [47]; when overproduced it can induce lymphoid malignancy in tumorigenic mice [48]. What is apparently reflected is that a given gene can cooperate with other genes in alternative combinations, each leading to a different consequence. Since human genetically cancer-prone cells are presumably preneoplastic, the question arises as to whether a tumor-promoting stimulus would produce in these cells a progression to or toward a neoplastic state. As shown in Figure 2, transfection of basal cell nevus syndrome fibroblasts, but not age-, race-, and sex-matched normal fibroblasts with mouse *pro*-1 produced a substantial extension of lifespan of some 20 population doublings after the point at which control untransfected BCNS cells or transfected normal cells senesced [49]. The DNA transfer showed similar efficiency

TABLE II. Characteristics of Cloned Mouse *Pro*-1 and *Pro*-2

	Pro-1	*Pro*-2
Length of minimum biologically active DNA sequences	1.05 Kb	3.7 Kb
Homology to other known sequences	Homologous to inverted complements of mouse Bam 5 repeat mouse Alu B_1 repeat	No known homologies
Estimated copy no.		
Mouse genome	$\sim 10^5$	Single copy
Human	~ 10	Single copy
Length of maximal ORF with pol II signals	195 nucleotides	Not known
Introns present	No	Yes
Evidence for homologous RNAs (transcripts)	Yes, in both basal and TPA-induced P^+ cells	Yes, in mouse skin carcinomas

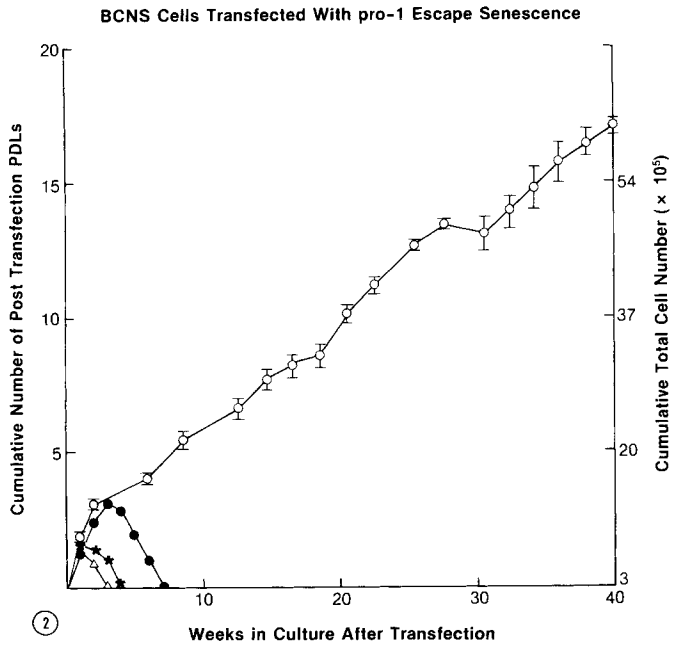

Fig. 2. Lifespan extension in human cancer-prone cells produced by transfection of *pro*-1. Transfection was performed as described [37,49]. BCNS GM 2098 cells transfected with *pro*-1 clone 26 [37] (○—○), BCNS cells transfected with inactive carrier DNA plasmic (★—★), nontransfected BCNS cells (●—●), paired normal GM 2912 cells transfected with *pro*-1 gene (△—△). For BCNS cells transfected with *pro*-1 each point represents the cumulative number of population doublings (PDLs) as well as the cumulative number of cells for the time in culture. Each point represents the mean for duplicate transfections with the range of the two values indicated by vertical bars through the points. In the three cases in which lifespan extension was not observed, the results are expressed only as the cumulative number of cells. Similar results were obtained with two additional sets of duplicate transfections.

in the BCNS and normal cells [49]. Thus *pro*-1 can apparently cooperate (in the absence of TPA) with BCNS gene(s) to produce partial immortalization. Addition of TPA or various growth factors such as EGF or PDGF to the *pro*-1 transfectants did not produce progression to a neoplastic endpoint [49]. Perhaps additional activated genes are required.

HUMAN NASOPHARYNGEAL CARCINOMA AS AN ANALOG OF TPA-TRANSFORMED JB6 P^+ CELLS: ACTIVATED HUMAN *PRO*-1

We have recently reported that DNA from tumorigenic anchorage-independent transformants ($T^3$6274 and RT101) derived by exposure of JB6 P^+cells to TPA can confer anchorage independence when transfected into JB6 P^+ cells [50]. This transforming activity appears to be determined by a gene(s) separate from *pro* genes. The DNA from these mouse transformed cells can not only transfer transforming activity into JB6 P^+ cells but can also transfer P^+ activity into JB6 P^- cells [50], thus suggesting that both genes for induction (*pro* genes) and genes for maintenance (transforming genes) of the neoplastic phenotype must be present in activated form in the DNA of tumorigenic derivatives of JB6 P^+ cells. Like mouse transformants $T^3$6274 and RT101, these nasopharyngeal carcinoma cells produce carcinomas when

injected into nude mice. In the case of CNE_1 and CNE_2 cells, the tumors are moderately to well-differentiated squamous carcinomas, whereas the carcinomas produced by RT101 or T^36274 are undifferentiated. Recent evidence has shown that the DNA from human nasopharyngeal carcinoma cell lines (CNE_1 and CNE_2) can transfer to mouse JB6 recipients either P^+ activity [46] or transforming activity (not shown).

To determine whether this CNE DNA might harbor activated homologs of mouse *pro*-1 or *pro*-2, a genomic library of CNE_2 was constructed and screened to retrieve homologs of both *pro*-1 and *pro*-2 [46]. CNE_2 *pro*-1, but not CNE_2 *pro*-2, turned out to be activated for P^+ activity. Figure 3 shows the specific activity of an activated CNE_2 cloned *pro* gene in comparison with cloned mouse *pro*-1 homolog from a normal human library. Human CNE_2 *pro*-1 clone i showed P^+ molar specific activity comparable to that of activated mouse *pro*-1, while the *pro*-1 homolog from a normal human library showed no P^+ activity (Fig. 3). Whether activated *pro*-1 plays a role in the etiology of nasopharyngeal carcinoma and how human *pro*-1 is activated are subjects of ongoing investigation. A working hypothesis is that activated *pro*-1 in concert with a tumor promoter acts to switch on constitutive expression of a transforming gene, which maintains the tumor cell phenotype in both mouse JB6 P^+ transformants and in human nasopharyngeal carcinoma cells.

PROMOTION-RELEVANT SIGNAL TRANSDUCTION: C KINASE SUBSTRATES AND OTHER PROTEINS

Since JB6 P^- cells are defective in a process that begins with the tumor promoter-receptor interaction and ends with the generation of a tumor cell endpoint,

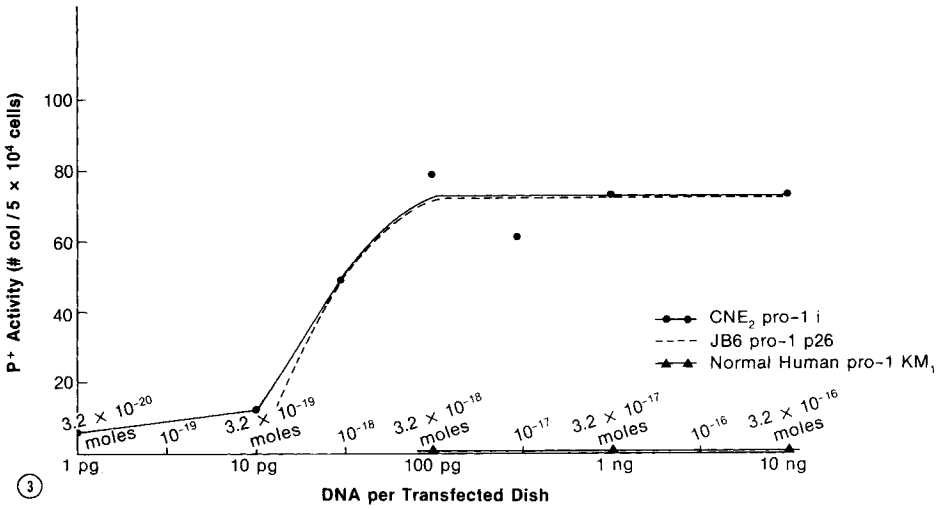

Fig. 3. *Pro*-1 homolog from nasopharyngeal carcinoma but not from normal human cells transfers P^+ activity. The P^+ assay was carried out as described [37]. One picogram to 10 ng CNE_2 *pro*-1 i DNA per dish together with 15 g carrier DNA from P^- recipient cells was transfected into P^- Cl 30 cells by calcium phosphate precipitation, followed by assay of TPA-inducible anchorage-independent colonies. Results are expressed as number of TPA-induced P^+ DNA-dependent agar colonies per 5×10^4 cells. The amount of CNE_2 DNA transfected is also expressed on a molar basis. Each point is the mean of duplicate agar dishes after transfection with CNE_2 *pro*-1 in a single experiment. Similar results were obtained in two additional experiments. For comparison the dashed line (points not shown) represents data for JB6 C1 22 *pro*-1 clone p26 [37]. Amount of mouse p26 DNA is plotted on a molar basis only.

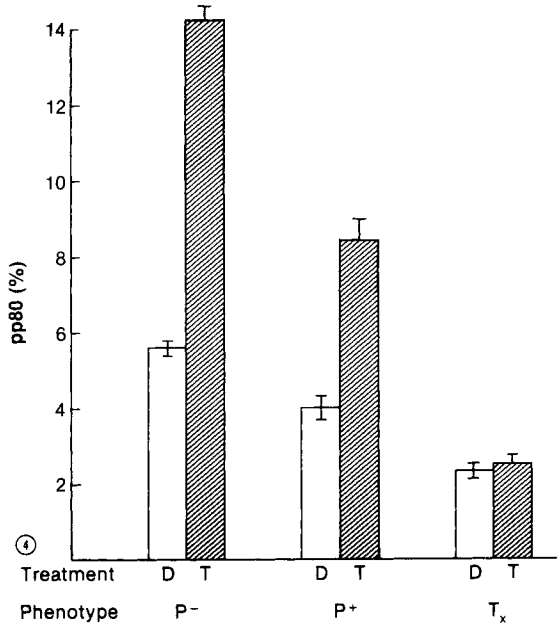

Fig. 4. Inverse relationship between levels of pp80 and stage of neoplastic progression. Cells were labelled for 1 hour in the absence or presence of TPA (10 ng/ml). Triton-X extracted >95% of pp80; results are expressed as a percentage of Triton-soluble cpm. Samples were analyzed by 12% SDS PAGE polyacrylamide gel electrophoresis. Each point represents the mean ± standard deviation of two or more lines of the same phenotype evaluated in two independent experiments (D, DMSO; T, TPA).

such P^- cells might turn out to be defective at any one or more steps from the cell surface receptor binding to various cytoplasmic second messenger events (signal transduction) to altered gene expression in the nucleus. We have found that the P^+ and P^- cells show no significant differences in the phorbol ester receptor number or binding affinity [51] or in C kinase activation or substrate availability [21,52]. Since transfer of an activated *pro* gene to P^- cells is sufficient to confer promotion sensitivity [37], the P^- cells are apparently not defective in any signals necessary for activated *pro* genes to function. Since there are undoubtedly other genes cooperating with *pro* genes to bring about transformation, the possibility arises that *pro* gene products might constitute signals for inducing other genes.

Differences in P^+ and P^- cells can be distinguished at the level of C kinase substrate phosphorylation [52,53]. Both basal and TPA-inducible levels of an 80-kD phosphoprotein (pp80) were highest in P^- cells, intermediate in P^+ cells, and nondetectable in transformed cells (Fig. 4). Thus, although P^- and P^+ cells both showed TPA inducibility, there was a relationship between pp80 levels and progression to neoplastic transformation. This inverse relationship between pp80 levels suggests that this phosphoprotein may play a role in maintaining a preneoplastic phenotype, and thus may be transformation relevant. Perhaps the loss of pp80 allows induction to a neoplastic phenotype.

One should note that phosphoproteins that mediate TPA-induced signal transduction and that may account for the promotion sensitivity of the P^+ phenotype do not necessarily have to be substrates for PKC. The P^+ and P^- phenotypes can also

be distinguished by the P^+-specific TPA-induced synthesis of two proteins of 15 and 16 kD molecular weight [54]. These proteins are localized in the nucleus and show maximum induction at 20 hours.

PROMOTION-RELEVANT SIGNAL TRANSDUCTION: LANTHANIDES AS TOOLS FOR UNDERSTANDING CALCIUM-REGULATED EVENTS

One interesting phosphoprotein has been identified that is a PKC substrate and is also sensitive to lanthanum, a transformation promoter that is a pharmacological analog of calcium but does not activate PKC [52]. Lanthanides readily induce neoplastic transformation in JB6 P^+ cells (with colony yields comparable to those with TPA of about 2,500 colonies per 10^4 cells) (Fig. 5). Lanthanides also induce a response in the promotion-resistant cells that is about 20% of the response of P^+ cells (in contrast to TPA and other promoters that induce only about 1% of the P^+ response in P^- cells). We have found that lanthanides must promote transformation by a mechanism other than C kinase activation. Although lanthanides will substitute for calcium in activating partially purified protein kinase C, these agents failed to activate PKC in intact JB6 cells, as measured by four independent experimental methods [52]. We have found, however, that there is a C kinase substrate (23 kD) found in both P^+

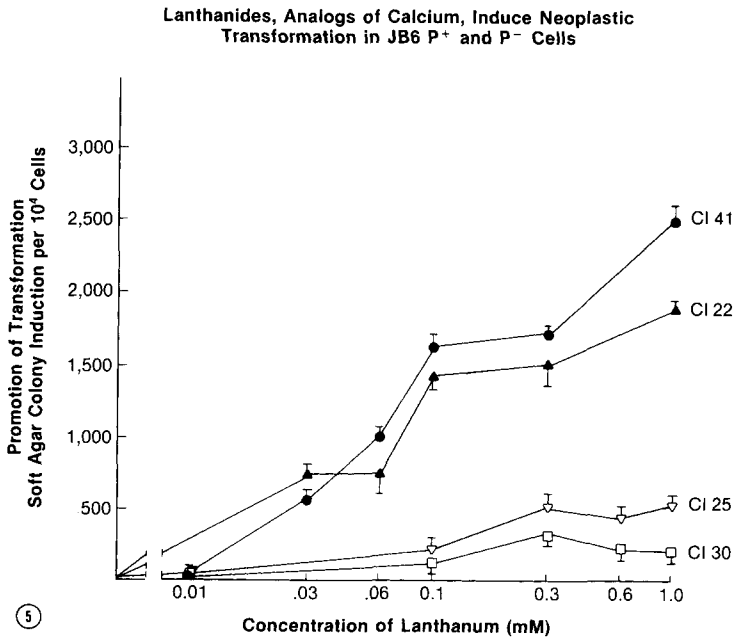

Fig. 5. Lanthanide induction of neoplastic transformation in JB6 P^+ and P^- cells. TPA-resistant (P^-) or TPA-sensitive ($^+$) preneoplastic JB6 cell lines growing in logarithmic phase in monolayer culture were trypsinized (0.03% trypsin) and suspended in 0.33% agar medium containing 10% serum and DMSO or lanthanum. This agar suspension (1.5 ml containing 10,000 cells/sample) was layered over a bottom agar of 0.5% that contained the same concentration of DMSO or lanthanum. Anchorage-independent colony induction is expressed as number of colonies greater than eight cells in size induced per 10^4 cells. Each point is the mean of at least three independent experiments each run in duplicate. Results are expressed as the mean ± standard error. Open symbols denote P^- lines; closed symbols are P^+ lines.

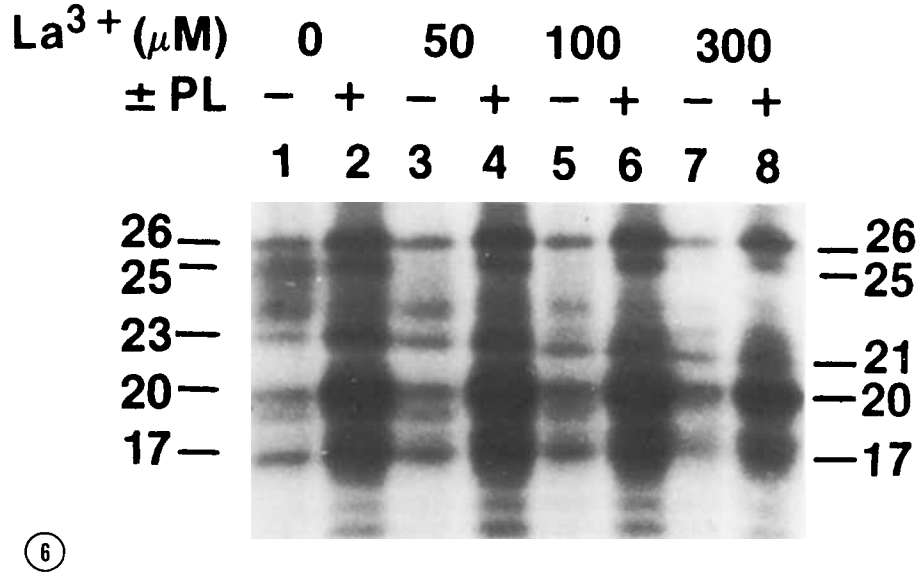

Fig. 6. Lanthanum sensitivity of a protein kinase C substrate. JB6 cell lysates were sonicated in Tris-HCl (20 mM) containing chelators (5.0 mM EGTA and 2.0 mM ethylenediamine tetraacetic acid) and centrifuged to remove nuclei and any remaining intact cells. The supernatant was centrifuged at 100,000g for 1 hour. The recovered supernatant was the cytosolic fraction and contained the calcium and phospholipid-dependent activity. The cytosolic fractions were incubated with required cations and ^{32}P-ATP in the presence or absence of phospholipid, as indicated. Calcium and phospholipid-dependent phosphoproteins are indicated at molecular weight 26, 25, 23, 20, and 17 kD. Lanthanum, from 50 to 300 μM, was added prior to initiation of the reaction. Note that phosphorylation of the 23kD protein is calcium and phospholipid dependent, and that the 23 kD phosphoprotein exhibits increased migration in the presence of lanthanum. Reprinted with permission [52].

and P^- cells that shows a band shift in response to lanthanum. Both P^+ and P^- cells have up to 16 protein kinase C substrates (Smith et al, unpublished); only this one exhibits any change in the presence of lanthanum. Increasing concentrations of lanthanum produce an increased migration of a 23kD PKC substrate to 21 kD in sodium dodecyl sulfate (SDS) polyacrylamide gels (Fig. 6). P^- cells showed a much smaller band shift.

The 23/21-kD lanthanum-sensitive protein kinase C substrate may represent a convergent, coincidental event on the promotion pathways of TPA and lanthanum. How might this potentially promotion-relevant PKC substrate act in the promotion of transformation process? The sensitivity of this substrate to lanthanum suggests a calcium link. Lanthanides substitute for calcium in numerous systems because of their high affinity for calcium-binding sites (10–1,000-fold higher than calcium) [55]. The 23/21-kD protein may in fact be a calcium-binding protein. Calcium-binding proteins in this molecular weight region are PKC substrates and are also known to exhibit altered electrophoretic mobilities in the presence of cations [56,57]. In fact, we know that calcium-regulated events are implicated in TPA-promoted neoplastic transformation. Employing chelators or calcium-deficient medium produces an almost complete

inhibition of TPA-promoted transformation, an event that is reversible upon addition of calcium [58].

MODELS FOR INTERACTION BETWEEN SIGNALS AND GENES

It appears that activated *pro* genes require calcium to function. Recent evidence has shown that in both parental P^+ cells and in *pro*-1 and *pro*-2 transfectants calcium depletion inhibits TPA-promoted transformation (Colburn et al, unpublished). Also, lanthanum induces transformation with a magnitude comparable to that observed in P^+ parental cells.

How might signals such as those described collaborate with *pro* genes? One possibility might involve a process by which TPA triggers a set of signals that induce *pro*-gene expression. These signals are expected to be identical in P^+ and P^+ cells (since P^- cells are competent recipients for activated *pro* genes). The products of activated and nonactivated *pro* genes are different and determine whether a neoplastic endpoint is reached. These products may function as differential signals for inducing other genes such as separate transforming genes [50]. The *pro* gene products might be DNA-binding proteins that regulate a transcriptional promoter. A calcium-binding protein or the nuclear p15 and p16 discussed above might be candidates for such a DNA-binding protein.

Alternatively, TPA may trigger a set of signals that induce not *pro* gene expression but the expression of other genes that cooperate with constitutively expressed levels of *pro* genes. The possible status of *pro* gene products as DNA-binding proteins could be similar to that discussed above.

GENES THAT DETERMINE SENSITIVITY TO TUMOR PROMOTION: WHAT FUNCTIONS DO THEY SPECIFY?

The above described results have made it clear that a dominantly acting single gene can confer sensitivity to promotion of neoplastic transformation by phorbol esters or various hormones. This gene can be an activated *onc* gene such as H-*ras* or one of several genes known to confer an "immortalizing" function such as c- or v-*myc*, polyoma large T, or adenovirus-5 E1a. Or this gene can be one of the recently described promotion sensitivity or *pro* genes that shows no homology to any known *onc* gene or other gene at the DNA level. It is of interest that v-*myc* transfers promotion sensitivity to JB6 promotion-insensitive cells with the same specific activity as *pro* genes (Shimada and Colburn, unpublished).

Can the promotion sensitivity function consist of immortalization? Probably not. Numerous spontaneously immortalized cell lines including mouse 3T3, 10T1/2, and prepromotable (prepassage 35) JB6 cells are not promotion sensitive [32,36]. Likewise, the promotion sensitivity function(s) appears not to simply consist of resistance to terminal differentiation. Differentiation-resistant, putatively initiated keratinocyte cell lines have not as yet been demonstrated to be promotion sensitive (Hennings, personal communication). Perhaps a clonal subpopulation of these cells will turn out to be promotable.

The promotion sensitivity found in mice sensitive to initiation-promotion carcinogenesis appears to be consistently associated with a sustained epidermal hyperplasia response to tumor promoters [7]. The promotion sensitivity found in JB6 P^+ cells is

consistently associated with a decreased synthesis of ganglioside G_T and a decreased activity of the superoxide anion-removing enzyme superoxide dismutase [1,34,36]. Both of these biochemical responses are dissociable from mitogenic response. Either of these might function as signal transducers for modulating gene expression. Synthesis of pp 80 and of 15 kD/16 kD proteins as well as a band shift in a 21-kD putative calcium-binding protein, discussed above, also distinguish P^+ from P^- cells and may function as signal transducers for regulating gene expression. Such P^+-specific events could be consequences of *pro* gene expression. As for the function(s) related to promotion sensitivity that are specified by activated H-*ras*, immortalization is probably not involved, since with few exceptions [59] immortalization or establishment appears not to be achieved with this gene. Whether an adenylate cyclase modulation via "G" proteins is involved is not clear [60].

ARE INITIATORS AND PROMOTERS ACTING ON THE SAME OR DIFFERENT GENETIC LOCI WHEN THEY PRODUCE CHANGES INVOLVED IN THE PROCESS OF PRENEOPLASTIC PROGRESSION?

The central dogma of tumor promotion has in the past held that promotion works only on initiated cells, not on normal or near normal cells that show only transient responses to tumor promoters. This suggested the possibility that tumor promoters might regulate the expression of genes mutated during the initiation event to produce preneoplastic progression. Several recent findings call for a re-examination of this assumption. The finding that mice bred for sensitivity to initiation-promotion skin carcinogenesis have apparently been bred specifically for promotion sensitivity [61–63] suggests that a gene for promotion sensitivity can be inherited independently of whether there exists an activated "initiation" gene. (Even if during the breeding the epidermis contained cells with activated "initiation" genes, such genes would not have been inherited in the germ line.) If promotion-sensitivity genes can be inherited independently of the presence of activated initiation genes, this suggests the possibility of two (or more) separate genetic loci. Another line of evidence that suggests separate genetic loci is that reported by Furstenberger et al, who found that first-stage tumor promotion can be achieved before—even 6 weeks before—initiation [64].

Evidence on gene cooperation [65,66] in transformation suggests 1) that two or more separate genes can cooperate or complement each other to produce a tumor cell and 2) that there is not an obligatory sequence for events that add up to neoplastic transformation. These experiments include the demonstration that *myc* and activated H-*ras* oncogenes can function together but not separately to transform embryo fibroblasts after transfection [65]. The *myc* function can alternatively be provided by other genes such as adenovirus E1a or polyoma large T. If these cooperating genes specify initiating and promoting events, respectively, in these cells, then separate loci are clearly involved. Balmain et al (this UCLA Symposia volume) have demonstrated that activated H-*ras* can function as an initiator of mouse skin carcinogenesis. The identity of the cooperating genes in this case is not clear, but they are presumably genes whose expression is elicited by tumor promoter exposure. In the case of *pro* genes, one can deduce (Table III) that they must be cooperating with gene(s) (in P^- recipient cells) other than activated Ha-*ras* since V-Ha-*ras* will completely transform JB6 P^- recipient cells without a requirement for activated *pro*-1 or *pro*-2 [58]. In this regard the JB6 P^+ cells may resemble "spontaneously" initiated human preneoplastic

TABLE III. Possibilities for Gene Cooperation in Multistage Carcinogenesis*

Initiation	Promotion
ras, mutated (activated)	*myc*, overexpressed
myc	*ras*
? (Non-*ras*)	*pro* + other genes that are promoter-inducible
? (Non-*ras*)	*myc* + other genes

*Two or more genes in altered or overexpressed form cooperate to produce cancer. The sequence of expression may not be obligatory.

cells. Possibilities for TPA-inducible genes that may cooperate with activated *pro* genes (Table III) can be expected to emerge from the characterization of a hybrid selected cDNA library of TPA-induced JB6 P^+ cells reported by Smith and Denhardt (this volume). Other cooperating gene expression events may be elucidated by analysis of the "mal" genes isolated from a skin carcinoma cDNA library by Krieg and Bowden (this UCLA Symposia volume).

A final suggestion for a "separate gene-nonobligatory sequence" mechanism has been set forth by zur Hausen and coworkers [67,68] who suggest that one route to cervical carcinoma in women involves expression of Herpes virus sequences as initiator and of certain human papilloma virus genes as promoters. The Herpes virus expression produces DNA alterations that are characteristic of responses specifically elicited by chemical carcinogens, not tumor promoters, and can be complemented to produce carcinomas by various agents known to show tumor-promoting activity such as certain hormones [67]. Frequently the papilloma virus expression occurs prior to the Herpes virus expression [67,68], suggesting that a constitutively promoted state can be attained independently of initiation and there may not be an obligatory sequence of events.

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