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Responses of Preneoplastic Epidermal Cells to Tumor Promoters and Growth Factors: Use of Promoter-Resistant Variants for Mechanism Studies

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The JB6 mouse epidermal cell model system is being used to study the mechanism of promotion of transformation. Promotion of anchorage independence in JB6 cells occurs in response to second-stage but not first-stage promoters, and is inhibited by inhibitors of second-stage not first-stage promotion. A number of variants that are resistant to the phorbol diester TPA have been derived. Some are resistant to plateau density mitogenic stimulation by TPA; others are resistant to promotion of anchorage independence by TPA. Some of the mitogen-resistant variants were promotable by TPA, thus ruling out a requirement for TPA mitogenesis in promotion of transformation in JB6 cells. TPA promotable clones were also sensitive to mezerein and EGF while the TPA nonpromotable variants were also resistant to mezerein and EGF, suggesting that sensitivity to promoters in these JB6 cells is determined at a level distal to receptor binding. Promotion sensitivity did not require available EGF receptors since two TPA promotable variants were EGF receptorless. The mitogenic response of JB6 cells to TPA may however be mediated by EGF since four of four mitogen-resistant variants showed low to zero levels of EGF binding. Tumor promoting phorbol esters produce specific changes in cellular gangliosides. Certain of these changes occur in promotable but not nonpromotable variants of JB6 cells, suggesting that ganglioside changes may be involved in the process of promotion of transformation.

Key words: TPA-resistant variants, promotion of anchorage independence, gangliosides, growth factor receptors

The "promotable" mouse epidermal cell line JB6 is being used as a model in our laboratory for studying the mechanism of late-stage promotion. JB6 cells respond to tumor promoting but not nonpromoting phorbol diesters by undergoing a progression to tumor cell phenotype as measured by anchorage independence and tumorigenicity [1,2]. The process occurs irreversibly [1] by a mechanism involving

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induction of new phenotypes [3]. Promotion of anchorage independence in JB6 cells also occurs in response to nonphorbol promoters including mezerein, cigarette smoke, detergents, and epidermal growth factor (EGF) [4].

The question of what promoter-induced events are required for promotion of transformation in vitro or in vivo is unanswered, though numerous biochemical and cellular responses to promoters have been described [5]. The results presented in this report suggest that neither promoter-induced mitogenesis nor EGF binding is a required event in promotion of transformation by 12-0-tetradecanoyl-phorbol-13-acetate (TPA) in JB6 cells. Promotion of transformation may, however, require specific ganglioside synthesis changes.

MATERIALS AND METHODS

Materials

Phorbol diesters were obtained from Chemical Carcinogenesis, Eden Prairie, Minn; cis and trans Pt, from Dr. Mathews Bradley of Merck, Sharpe, and Dohme; A23187, from Dr. Henry Hennings of NCI; and epidermal growth factor (EGF), from Collaborative Research. D[1-^{14}C]glucosamine HCl (55 mCi/mmole) was procured from Amersham Searle and ^{125}I-EGF (110 μCi/μg) from Kor Biochemicals.

Promotion of Anchorage Independence in JB6 Cells

Mouse epidermal JB6 cell lines [6] were exposed to tumor promoters in 0.33% agar medium and colonies enumerated as described previously [1]. For antipromoter studies, the inhibitor was added to cells simultaneously with promoter in agar.

Plateau Density Mitogenic Response Assay

Mitogenic response was determined by exposing cells at plateau density to TPA in medium containing 5% fetal calf serum as described [6,7] and determining the cell number increase in response to TPA.

Selection for JB6 Variants Resistant to Plateau Density Mitogenic Stimulation by TPA

The promotable JB6 C141 was exposed after reaching plateau density to TPA and colchicine in a selection procedure described elsewhere [6,7] which is analogous to that described by Pruss and Herschman [8] for producing EGF-resistant variants of 3T3 cells. The cells that showed a mitogenic response were trapped in mitosis by colchicine, detached, and washed off. The remaining cells were carried through two to six selection cycles and cloned.

^{125}I-EGF Binding Assay

^{125}I-EGF binding assay and Scatchard analysis were performed according to the method of Magun et al [9].

Ganglioside Labeling and Isolation

The synthesis of individual gangliosides was determined by incorporation of D[1-^{14}C]glucosamine into cellular gangliosides followed by chloroform: methanol extraction and separation by thin-layer chromatography as described [21]. Exposure to 10 ng/ml (1.6×10^{-8} M) TPA was carried out for 24 h with 4-h terminal incorporation of precursor at 4 μCi/ml.

RESULTS

Table I shows the activity of a number of phorbol and nonphorbol promoters in inducing anchorage independence in JB6 C141 cells. The promoters that show activity as second-stage promoters in mouse skin as described by Slaga et al [10] also show activity in promotion of transformation in JB6 cells. These include the tumor promoting phorbol diesters, which act both as first- and second-stage promoters, and mezerein, which acts primarily as a second-stage promoter. The first-stage promoter, the calcium ionophore A23187 was not active in promoting transformation in JB6 C141 cells. In addition, cis and trans diaminedichloroplatinum and hydrogen peroxide, which show sister chromatid exchange-inducing activity, and the growth factors EGF and a human transforming growth factor (TGF) described by Todaro et al [11] showed activity for promotion of anchorage independence.

Another characteristic of promotion of anchorage independence in JB6 cells is that it is inhibitable by second-stage but not first-stage antipromoters as described by Slaga et al [12]. As shown in Table II the second-stage antipromoter retinoic acid inhibited promotion of transformation by TPA but the antiproteases antipain and leupeptin, which inhibit first-stage promotion, did not, even at concentrations greater than or equal to those that inhibited promotion of transformation in 10T1/2 cells [13]. Antiproteases TLCK and TPCK also showed little or no inhibition of promotion of anchorage independence in JB6 cells (not shown).

We then sought to use this model for late-stage promotion to discern required events in the process. We have recently described an approach to testing the possibility that promoter-dependent mitogenic stimulation is required in promotion of transformation [6,7]. This approach involved selecting promotable JB6 cells for re-

TABLE I. Anchorage Independence Induced in Mouse JB6 Cells by Second-Stage, Not First-Stage Promoters*

Promoter	Stage (in mouse skin)	Concentration	Colony yield (No. per 10^4 cells-bkg) 10%	20%
TPA	1,2	1.6×10^{-8}M	2,192	3,714
PDBz	1,2	1.6×10^{-8}M	– –	2,488
PDBu	1,2	1.6×10^{-8}M	24	1,848
Mezerein	2	1.6×10^{-8}M	78	2,352
CisPt	?	8.3×10^{-7}M	– –	530
TransPt	?	1.7×10^{-5}M	– –	952
H_2O_2	1,3	9.8×10^{-6}M	– –	820
A23187	1	10–500 ng/ml	– –	0
EGF	?	1.6×10^{-9}M	183	1,862
TGF	?	5 μg/ml	1,515	120

*JB6 C141 cells were exposed to promoters in agar medium containing 10% or 20% fetal calf serum, and the number of colonies induced per 10^4 cells was determined as described previously [1]. The colony yield is expressed as the mean for two or three experiments run in duplicate minus the solvent control background. Background values averaged 15 colonies per 10^4 in 10% serum and 60 per 10^4 in 20% serum. The designation of the stage of promotion in which each compound is active is taken from Slaga et al [10 and personal communication]. The TGF was a partially purified transforming growth factor [11] extracted from the media in which A673 human rhabdomyosarcoma tumor cells were grown, and kindly supplied by G.J. Todaro and C. Fryling.

sistance to plateau density mitogenic stimulation by TPA using the procedure described by Pruss and Herschman [8] for selecting EGF-resistant 3T3 cells. A requirement for TPA-dependent mitogenesis would predict that the mitogen-resistant variants would be nonpromotable. As shown in Figure 1A, two cell lines, R219 and R23, were resistant to mitogenic stimulation by 1–100 ng/ml (1.5×10^{-9}–1.6×10^{-7}M) TPA. R6141 and R28 were also TPA mitogen resistant [7]. Fig. 1B shows that three of these four mitogen-resistant lines, R219, R6141, and R23 were promotable to anchorage independence by TPA. Thus, promotion of transformation by TPA in JB6 cells can occur without this release-from-quiescence type of mitogenic response to TPA.

Cross-resistance to the nonphorbol promoters mezerein and EGF occurs in two TPA promotion-resistant clones of JB6, C130, and C125 (Table III). This suggests that the basis for the resistance involves a defect distal to receptor binding rather than a phorbol ester receptor deficiency. This expectation was borne out by determination of phorbol diester receptor number and affinity as reported elsewhere [14,15].

TABLE II. Promotion of Anchorage Independence Inhibited by Inhibitors of Second-Stage, Not First-Stage Promotion*

Promotion inhibitor	Stage inhibited (in mouse skin)	Concentration	% Inhibition of TPA-induced anchorage independence
Retinoic acid	2	10^{-6}M	72 (22)
Antipain	1	50–200 μg/ml	0–10
Leupeptin	1	50 μg/ml	0

*Induction of anchorage independence by TPA (1.6×10^{-8}) was carried out as described [1] with or without simultaneous exposure to retinoid or antiprotease, and colonies were enumerated. Each value is the mean for 2-3 experiments run in duplicate.

TABLE III. TPA-Resistant Variants Are Cross-Resistant to EGF and Mezerein for Promotion of Anchorage Independence*

Cell line	Phenotype	Promoter	Concentration	Anchorage independence response
JB6 C121	M+P+	Mezerein	1.6×10^{-8}M	490
			1.6×10^{-7}M	920
JB6 C130	M+P−	Mezerein	1.6×10^{-8}M	70
			1.6×10^{-7}M	150
JB6 C141	M+P+	EGF	1.6×10^{-9}M	1,862
			8.0×10^{-9}M	3,560
JB6 C125	M+P−	EGF	1.6×10^{-9}M	0
			8.0×10^{-9}M	24

*Induction of anchorage independence assays were carried out as described in the legend to Table I using 20% serum in agar. JB6, C121 and C141 were mitogenesis sensitive and promotion of anchorage-independence sensitive to TPA (M+P+) (Fig. 1). JB6, C125, and C130 were mitogenesis sensitive and promotion resistant to TPA (M+P−).

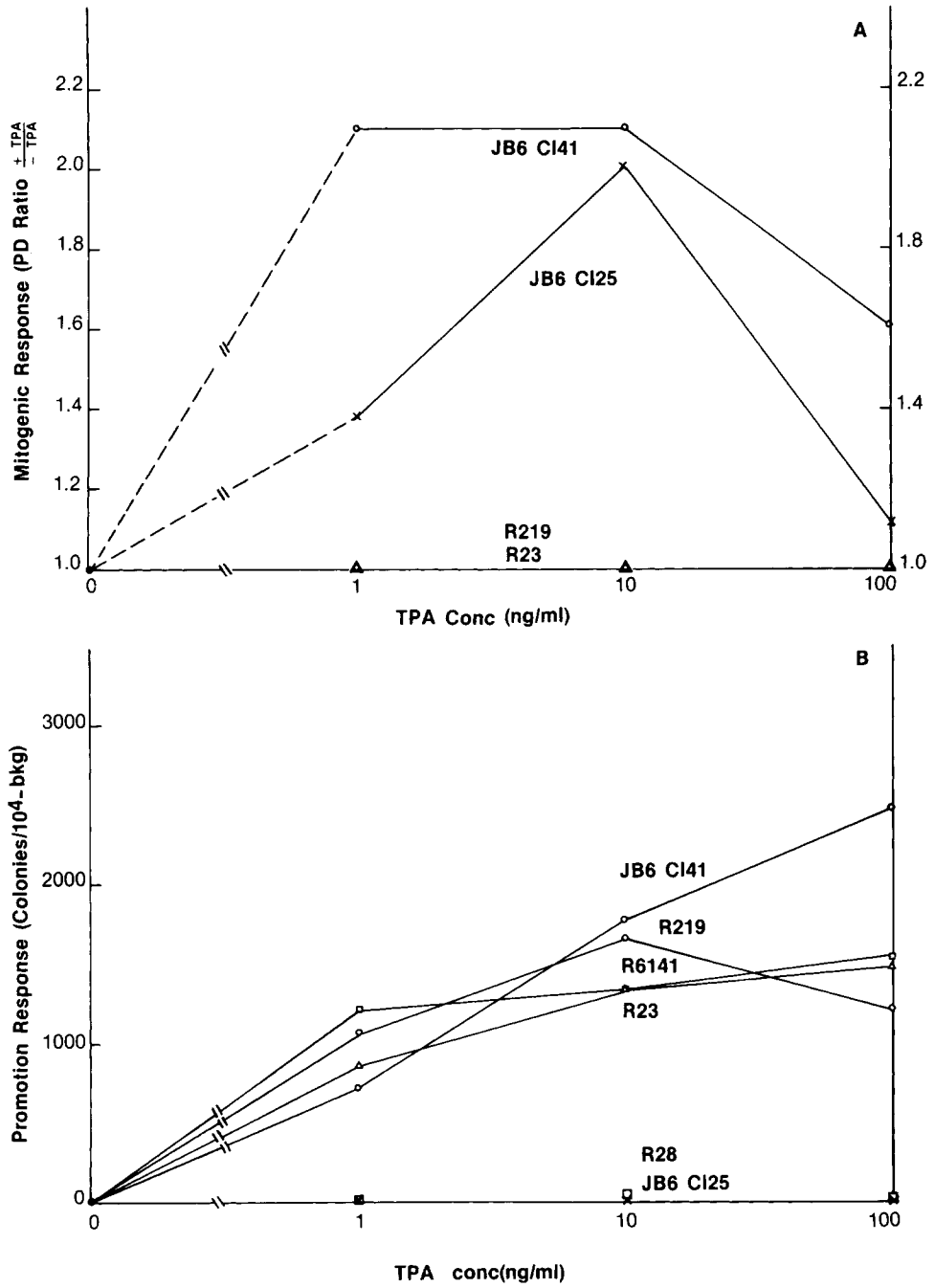

Fig. 1. Responses to TPA of sensitive and resistant JB6 variants: JB6 clones 41 and 25 were derived by nonselective cloning of JB6 [4] and R219, 23, 6141, and 28 by cloning after selection of JB6 Cl 41 for resistance to TPA mitogenesis [6,7]. A) Mitogenic response expressed as the ratio of plateau cell number in TPA treated: untreated cultures determined as described in Materials and Methods. B) Promotion of anchorage independence response [1] expressed as number of colonies per 10^4 cells induced in 0.33% agar medium by TPA minus the solvent control (bkg) value which ranged from 0 to 50 colonies per 10^4 cells. For mitogenic response, each value is the ratio of the cell number means for two flasks each of TPA treated and control. Duplicate flasks varied in cell number by 5–10%. Mitogenic response assays were repeated three times for each cell line at 10 ng/ml TPA. For promotion of anchorage independence assays, each value is the mean for duplicate dishes which varied by 10% or less. Promotion response assays were repeated six times for each cell line at 10 ng/ml.

The possibility that TPA action may be mediated by EGF or another growth factor that binds to EGF receptors was considered. If such is the case, TPA resistance might be attributable to an EGF receptor deficiency. Table IV shows that two *nonpromotable* cell lines, C125 and C130, showed moderate to high levels of EGF binding while three *promotable* lines, R219, R6141, and R23 showed little or no EGF binding. EGF binding can thus be ruled out as a requirement for promotion of transformation in JB6 cells by TPA. It is noteworthy that four of four TPA mitogen-resistant cells showed little or no EGF binding thus suggesting that EGF or a related growth factor may function to mediate the mitogenic action of phorbol esters.

Since TPA is known to produce a number of plasma membrane changes [5] and since gangliosides function in responses to a number of hormones or toxins [16–19], we investigated the role of TPA-induced changes in these sialic-acid-containing plasma membrane glycolipids. Figure 2 shows that TPA produced a tenfold decrease in precursor incorporation into a trisialoganglioside (G_T) and a twofold increase in incorporation into G_{D1b} and an unidentified ganglioside. As described elsewhere [20,21], these changes were completely blocked by simultaneous exposure to the antipromoter retinoic acid. Figure 3 shows that TPA-promotable variants consistently showed the G_T decrease and G_{D1b} increase in response to TPA, while the nonpromotable counterparts did not. Since TPA produced a 50% decrease in total ganglioside synthesis the promotable lines showed an *absolute* decrease in GT of 20-fold and little or no change in G_{D1b}. Similarly, the nonpromotable cell lines showed no absolute change in G_T but a substantial decrease in G_{D1b}.

DISCUSSION

These studies on the mechanism of promotion of anchorage independence in JB6 cells lead to several conclusions, which are summarized in Table V. Events that appear not to be required include promoter-induced mitogenic stimulation from quiescence and growth factor binding to EGF receptors. In addition, we have pre-

TABLE IV. EGF Receptor Levels in TPA-Resistant Variants*

Cell line	Phenotype	^{125}I-EGF binding DPM/10^6 cell[a]	Nbr BS/cell $\times 10^{-3}$
JB6 C141	M+P+	3160 ± 118	60
JB6 C122	M+P+		37
JB6 C125	M+P−		8
JB6 C130	M+P−	6150 ± 92	
R219	M−P+	17 ± 20	[b]
R6141	M−P+	212 ± 24	[b]
R23	M−P+	57 ± 60	[b]
R28	M−P−	20 ± 7	[b]

*Characterization of the phenotypes of JB6 variants was carried out as described [6,7]. Binding of ^{125}I-EGF and Scatchard analysis were determined as described [9]. Four hour binding at 4°C was determined. Each value is the mean for three separate dishes. Nonspecific binding (< 1% of specific binding for cell lines having > 5,000 binding sites per cell) has been subtracted. BS: binding sites

[a] ^{125}I-EGF concentration: 0.5 ng/ml.

[b] Too low to determine

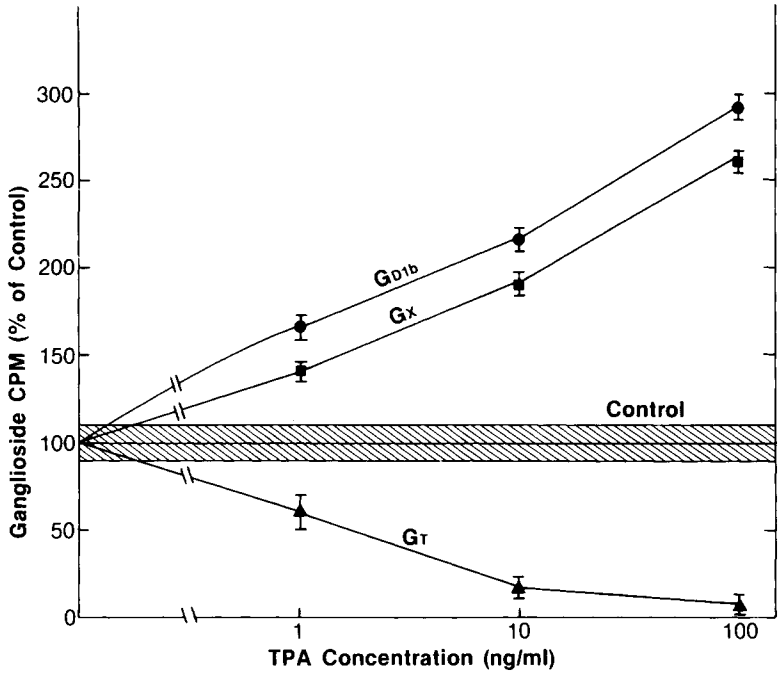

Fig. 2. Concentration dependence of changes in ganglioside synthesis in response to TPA. TPA-induced changes in gangliosides synthesis (CPM 1-^{14}C-glucosamine incorporated as % of control) was calculated as:

$$\frac{\text{G-associated CPM as \% of total ganglioside CPM Treated}}{\text{G-associated CPM as \% of total ganglioside CPM Control}} \times 100$$

where "G" stands for trisialoganglioside G_T the disialoganglioside G_{D1b} or G_x an unidentified ganglioside. Gangliosides were separated on precoated silica gel G plates (250 microns) with $CHCl_3$:CH_3OH:NH_3OH:H_2O (60:35:0.5:7.5 v/v). Plates were exposed to iodine vapors and ganglioside bands marked. After the iodine sublimed, the gangliosides were scraped, dissolved in $CHCl_3$:CH_3OH:H_2O (10:10:1 v/v/v and counted in a scintillation counter with hydrofluor at an efficiency of 50% for 1-ml samples. The radioactivity recovered from the plate was 80–90% of total activity applied. Ganglioside associated cmp recovered from control plates was approximately 2,000 cpm and, from TPA-treated, approximately 1,100 cmp for a typical experiment. The shaded area indicates the standard error of control values and the vertical bars, the range for duplicate TPA-treated samples.

viously found that promoter induction of ornithine decarboxylase cannot be involved because it does not occur in JB6 cells [22]. More recently, we have investigated the possible role of promoter stimulated hexose transport [23] and found that this response does not correlate with promotability when TPA-sensitive and TPA-resistant variants are compared [24]. Both hexose transport [24] and EGF receptors (Table IV) may, however, be involved in the plateau density mitogenic response to TPA.

We have recently reported on other molecular events that may be involved in promotion of transformation by phorbol diesters in JB6 cells. These include

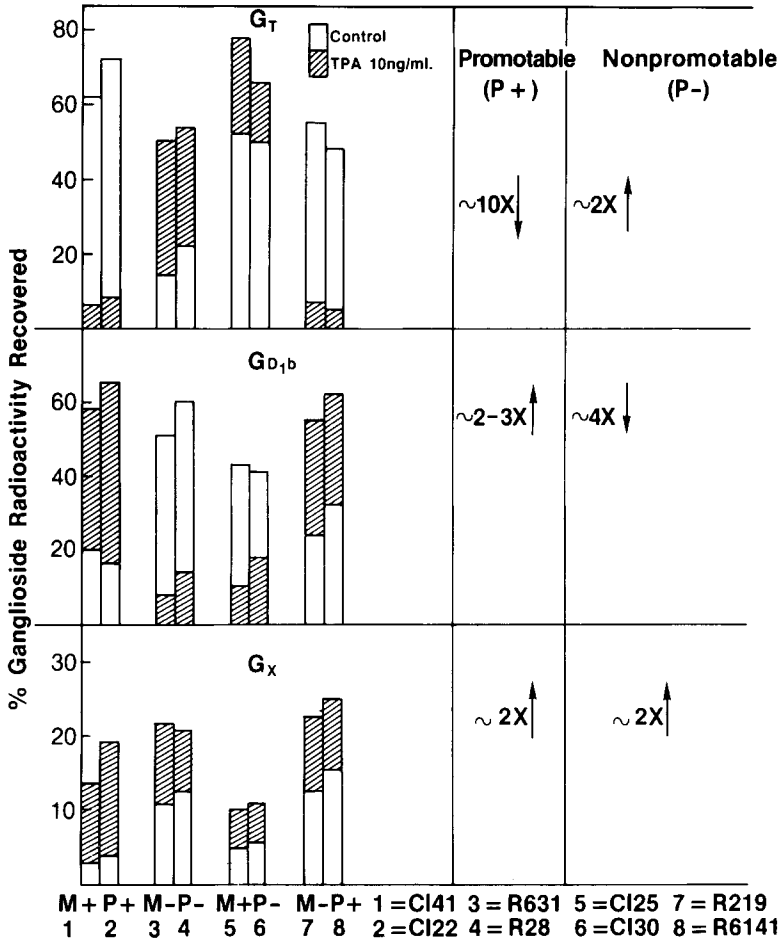

Fig. 3. Promotable but not nonpromotable variants show decreased G_T and increased G_{D1b} after TPA exposure. The TPA sensitive and resistant variants of JB6 cells were derived as described in the legend to Figure 1 or as described elsewhere [4,6,7]. The determination of presursor incorporation into individual gangliosides at 20-24 h TPA exposure was carried out as described in the legend to Figure 2. The radioactivity incorporated into each ganglioside was expressed as percent of total ganglioside associated radioactivity. Each value is the mean for duplicate flasks that varied by 5-10% from the mean. M +/−: Sensitive or resistant to plateau density mitogenic stimulation by TPA. P +/−: Sensitive or resistant to promotion of anchorage independence by TPA.

phorbol diester binding to specific binding sites in JB6 cells, an event that occurs in all promotable clones of JB6 tested to date [14,15] and decreased synthesis of collagen that occurs in promotable clones of JB6 and is antagonized by antipromoting concentrations of retinoic acid [25,26]. Alternatively, an event transcriptionally coupled to the regulation of collagen synthesis could be involved in the promotion process.

TABLE V. Mechanism of Promotion of Tumor Cell Phenotype in JB6 Epidermal Cells

Events not required	Events that may be involved
	Promoter binding to specific binding site [14,15]
Promoter-induced mitogenic stimulation from quiescence [6,7]	Early events in signal transduction triggered by receptor binding
EGF or other ligand binding to EGF receptor as mediator [14,15, Table IV]	Decreased procollagen [25,26] An event transcriptionally coupled to the procollagen switch
Promoter induction of ODC [22]	Specific shifts in ganglioside biosynthesis [20,21, Figs. 2 and 3]
Promoter stimulated hexose transport (24)	
Promoter induction of Proteases (see Table II)	

TABLE VI. Proposed Model for Sequence of Events Required for Promotion of Transformation in JB6 Cells

TPA + promotable JB6 cells	→	→	→	Tumor cell
	reversible ion fluxes or changes in plasma membrane enzymes	G_T↓ collagen↓ event coupled to collagen mRNA↓	irreversible changes in receptors, growth factor production, and/or cell communication	

In this report, we have demonstrated that specific promoter-induced changes occur in JB6 gangliosides, in particular a tenfold G_T decrease. These changes that are antagonizable by retinoic acid [20,21], occur in promotable but not nonpromotable variants of JB6, thus suggesting that gangliosides may be important in promotion of tumor cell phenotype. Specific gangliosides have also been implicated in responses to hormones such as serotonin [19] and thyroid stimulating hormone [18], in responses to toxins such as cholera toxin [16] and tetanus toxin [17] and even in responses to phorbol diesters in the case of phorbol-ester-induced differentiation of human melanoma cells [27]. Gangliosides and other glycolipids may be involved in the induction and maintenance of malignant transformation by viral and chemical carcinogens [28–32]. We have recently found that addition of G_T to JB6 cells during exposure to TPA blocks promotion of anchorage independence, thereby confirming the suggestion from the variant correlation that a G_T decrease could be involved in the promotion process.

Table VI presents a proposed model for the sequence of events that must occur during promotion of transformation in JB6 cells. All the events listed are reported responses to TPA in various cell systems [5]. The earliest events that may include ion or enzyme fluxes might be expected to be reversible, whereas at least some later events involved in maintenance of the tumor cell phenotype may be irreversible.

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