Phenotypic Expression of Transformation: Induction in Cell Culture by a Phorbol Ester

Abstract. *A biologically potent, tumor-promoting phorbol ester from* Croton tiglium L. *induces the appearance of transformed clones in a population of contact-inhibited fibroblasts of the mouse cell line 3T3. Mixed populations of cells with 10,000 3T3 cells and 100 virus-transformed cells were exposed to the phorbol ester and, in comparison with untreated cells, showed a marked increase in the numbers of transformed clones that grew. Exposure of 3T3 cultures to the carcinogen 7,12-dimethylbenz(a)anthracene alone or prior to exposure to the phorbol ester did not cause any increase in the number of transformed clones.*

The tumor-promoting activity of certain materials such as the phorbol esters of croton resin, as well as tobacco leaf extracts and cigarette tar, is known (*1*); however, the mechanisms by which these agents act are not well understood. In the usual assay system on mouse skin, a single exposure to a subcarcinogenic dose of an appropriate chemical carcinogen is followed by continuous exposure to promoting agents. This treatment results in the appearance of neoplastic skin lesions.

This situation may have a parallel in viral oncogenesis. Todaro and Green (*2*) reported that several generations of cell proliferation are required for the phenotypic expression of the transformed state in cultures of the 3T3 line of mouse fibroblasts infected with simian virus 40 (SV40), whereas one generation is sufficient to fix the transformed state.

A purified fraction of croton resin (CRA) was capable of enhancing the phenotypic expression of both spontaneous and virus-induced loss of contact inhibition in cultures of the 3T3 line of mouse fibroblasts. This suggested that a phenomenon similar to tumor promotion had been achieved in a cell culture system.

The 3T3 cells (*3*) were grown in Dulbecco's modification of Eagle's medium (*4*) in 50-mm plastic dishes. The loss of contact inhibition had been initially observed when the cultures had been passaged with a large inoculum (10,000 cells per dish) for several months. This procedure apparently selects for cells that have a reduced capacity to be contact-inhibited. However, until loss of contact inhibition occurred in untreated cells, CRA did not have any observable effect on 3T3 cells.

The effect of CRA on the outgrowth of clones of 3T3 cells that are no longer contact-inhibited is shown in Table 1 and Fig. 1, A and B. In experiment 1, designed to test the effects of several substances on cell growth, it was found that several dense colonies were present in a control plate and that continuous exposure of the cells to CRA increased the number of multilayered colonies more than threefold. Two subsequent experiments with five replicates per set confirmed the observation. 7,12-Dimethylbenz(a)-anthracene (DMBA), a very potent initiator and carcinogen, did not induce the growth of transformed clones at doses of 0.1 or 0.001 μg/ml.

The inoculum for experiment 1 was derived from a plate that had no gross indication of morphological transformation, whereas the inocula in experiments 2 and 3 were prepared from plates that contained a few colonies characteristic of transformed cells.

The ability of CRA to elicit the formation of transformed colonies was apparently independent of the state of growth of the culture. With a multiple treatment in which CRA was applied after a monolayer had been achieved, enhancement of the numbers of transformed colonies was readily apparent (experiments 2 and 3). Prior treatment with DMBA at a dose that was not appreciably toxic to growth (0.001 μg/ml) had no perceptible effect; however, exposure to the carcinogen at a toxic dose (0.1 μg/ml) caused a reduction of 50 percent in the number of colonies not contact-inhibited. These results suggested that CRA had enhanced the growth of certain transformed cells that would not otherwise have proliferated in a population containing an excess of contact-inhibited fibroblasts.

To determine whether CRA could enhance the growth potential of known transformed cells in a mixed population, 10,000 untransformed 3T3 fibroblasts and 100 virus-transformed cells were cultivated together in the absence or presence of CRA (1.0 μg/ml) with

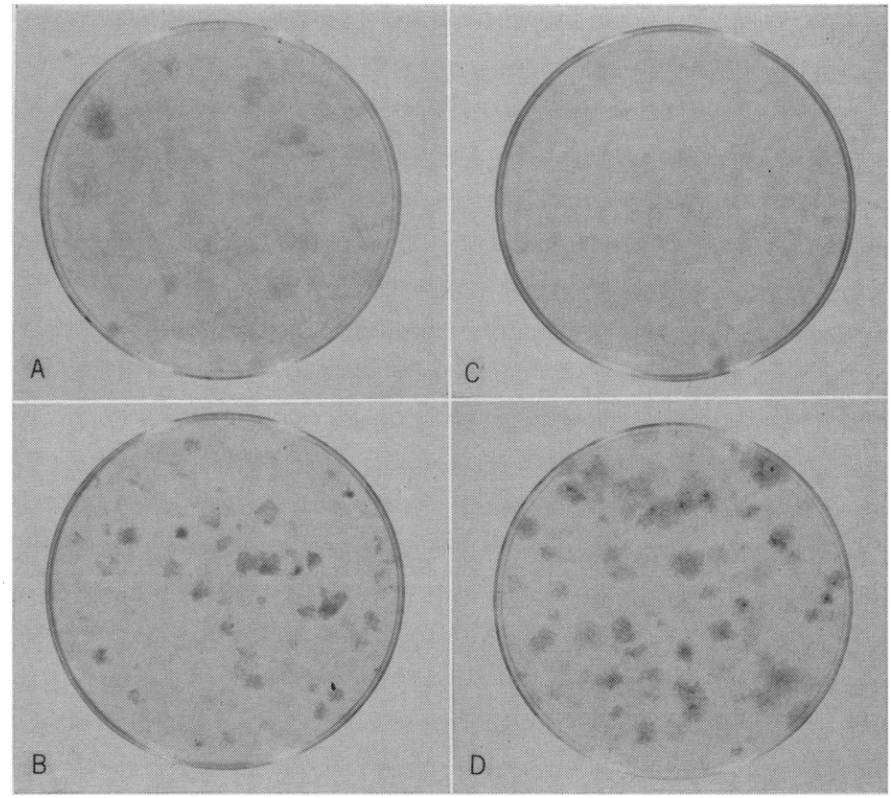

Fig. 1. Cultures of 3T3. (A) 3T3 control; (B) 3T3 with CRA (1.0 μg/ml); (C) 10,000 3T3 cells and 100 virus-transformed cells, control; (D) as in (C) with CRA (1.0 μg/ml). Cultures were fixed with methanol and stained with Giemsa.

Table 1. Effect of CRA and DMBA on growth of transformed clones of 3T3 cells. The CRA concentration was 1 μg/ml. The DMBA concentration for experiments 1 and 2 was 0.001 μg/ml; for experiment 3, it was 0.1 μg/ml. In experiment 2, treatment I lasted 16 days, and treatment II was for 17 days. In experiment 3, treatment I lasted 14 days, and treatment II was for 15 days.

Treatment	Transformed clones per plate (No.)*		
	Expt. 1	Expt. 2	Expt. 3
Control	16	9 (8–10)	8 (7–9)
CRA	55	33 (23–44)	43 (37–52)
DMBA	15		5 (3–7)
I-Control, II-CRA		20 (15–26)	56 (53–59)
I-DMBA, II-CRA		18 (16–20)	24 (20–29)

* Average of five plates; range of values in parentheses.

the use of four replicates per set. The virus-transformed cell line was SV40-transformed (*5*) and was plated 24 hours prior to the plating of the untransformed cells. The concentration of CRA had no inhibitory effect on the cloning efficiency or growth of mass cultures of 3T3 cells and caused a slight reduction (11 to 30 percent) in the cloning efficiency of SV40-transformed cells.

These experiments (Table 2) show that the growth potential of a virus-induced neoplastic cell in a population containing a 100-fold excess of untransformed cells is increased to 166 to 179 percent in the presence of CRA. On the other hand, the growth of SV40-transformed cells appeared to be somewhat inhibited by the 3T3 cells in the absence of CRA. Figure 1, C and D, shows a similar experiment with a line of cells transformed sequentially with polyoma and SV40.

The postulated structures of the purified, active tumor-promoting fractions from *Croton tiglium L.* (*1*, *6*) indicate that the molecule contains both hydrophobic and hydrophilic groups, and an interaction with biological membranes is a clear possibility. It is possible that these agents could affect the permeability properties of cell membranes, either by allowing materials not usually capable of entering the cells to enter, or by allowing cell components to leach out. Either or both of these processes could have an influence on control mechanisms of the cell, possibly resulting in tumor formation. It has been found that CRA and other promoting agents prepared in our laboratory result in a release of lysosomal enzymes in vitro from rabbit liver preparations (*7*).

Table 2. Effect of CRA on growth of SV40-transformed cells in mixed culture, as shown by number of transformed clones per 100.

SV40 cells alone			SV40 (100)+3T3 (10,000)		
I	II	III	I	II	III
			Control		
33	38	45	24	19	35
			CRA		
23	29	40	43	33	58
			CRA/control, percent		
70	76	89	179	174	166

The observation that cells in culture can be released from contact inhibition by a tumor-promoting agent that interacts with biological membranes suggests that there may be a similar mechanism in vivo. The primary role of a tumor promoter may be the alteration of the properties of cell membranes. One result is that cells are no longer contact-inhibited by their neighbors. A corollary of this model proposes that membrane properties of cells may exert an important controlling influence over the propagation of neoplastic cells in the presence of untransformed cells and that these membrane properties may have general importance for the induction of the sequence of events that leads to cell division. This theory is supported by the work of several investigators (*8*) who suggested that there may be an intimate relation between cellular contact and the biochemical events necessary for macromolecular synthesis and mitosis.

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Quartz: Extreme Preferred Orientation Produced by Annealing

Abstract. *Annealing of samples of flint under high pressure, after hot-working in the β-quartz stability field, produced an exceedingly strong concentration of c-axes parallel to the direction of compression. A specimen deformed under identical conditions, but not annealed, exhibited a much weaker orientation. The strength of the annealed orientation rivals that of the remarkable "cube texture" produced by annealing some face-centered cubic metals after extreme reduction by rolling.*

Quartz rocks deformed under metamorphic conditions in nature recrystallize into aggregates with moderate-to-high preferred orientations. The highest concentration of *c*-axes reported is 37.5 percent per 1 percent area (*1*). A comprehensive experimental study of the development of preferred orientation in quartz aggregates (*2*, *3*) has found that recrystallization of flint *during* compression under high temperatures and pressures produces two types of preferred orientations (Fig. 1) (*3*). The conditions under which these orientations are developed will be reported separately. Many recrystallized flints are too fine-grained for measurement with conventional universal-stage techniques and are being investigated with x-ray methods (*4*), together with photometric optical analysis (*5*), in my laboratory. These studies reveal orientations similar to those in Fig. 1.

In contrast to these weak-to-moderately strong preferred orientations produced in "syntectonic recrystallization," the extremely strong orientation that I now report was formed by *annealing* of a hot-compressed flint specimen at hydrostatic pressure. Two cylindrical samples of flint were shortened by 34 percent at 750°C, under a 6-kb confining pressure, at a strain-rate of 0.8×10^{-5} sec^{-1}, in the field of β-quartz stability. Specimen DT-459 (Fig. 2, left) was not annealed,