

Is the product of the *src* gene a promoter?

(Rous sarcoma virus/phorbol esters/chicken cells/collagen/viral carcinogenesis)

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Contributed by Melvin Calvin, October 23, 1978

ABSTRACT Addition of a potent promoter, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), to primary avian tendon or chicken embryo fibroblast cells infected with a temperature-sensitive mutant of Rous sarcoma virus produced a complete transformed phenocopy at the nonpermissive temperature by the criteria tested. While normal, uninfected cultures also shifted towards a transformed phenotype after TPA addition, they did not achieve the same degree of morphological and biochemical alteration seen in virus-infected, TPA-treated cells. It is proposed that viral carcinogenesis, despite its rapidity, may occur in two stages: an "initiation" step caused by expression of a part of viral genome other than *src* (or by integration) and a promotion step (itself a multistep process) caused by the activation of the *src* gene. The *src* gene product could be enhanced or replaced by other promoting agents.

We have gained tremendous knowledge of the structure and the genetics of RNA tumor viruses in recent years (1-3), yet we know relatively little about the mechanism(s) of viral carcinogenesis. The early optimism that viral transformation could provide a simple model for studying malignancy and that, once we identified the "oncogene" (4), we would understand cancer, has given way to skepticism and to an abrupt shift of interest from tumor viruses to other environmental carcinogens.

The rapidity and completeness by which RNA tumor viruses could transform cells in culture and the simplicity of the assay systems were the initial arguments for the use of RNA tumor virus-transformed cells as models for studying malignancy. These same reasonings, however, now are used to support the argument that viral transformation is not a valid model for most forms of cancer. Where chemical carcinogens play a role in cancer induction (purported to be the case in 80% or more of all human cancers) the development of tumors is slow and multistep. Viruses transform too fast and appear to do it in a single step. Studies with mutants of RNA tumor viruses have led to the postulate that the product(s) of one region of the viral genome termed *src* (sarcoma specific) is both necessary and sufficient for transformation in culture and tumor formation *in vivo* (for review see ref. 1). However, if indeed *src* is the "oncogene," its deletion or its absence from a viral genome should make the virus incapable of causing cancer *in vivo* or transformation in culture. Yet many "*src*-defective" viruses such as Rous-associated virus (RAV)-2, RAV-6, RAV-50, and avian myeloblastosis virus induce a variety of tumors *in vivo*, including lymphoid leukemia, myeloblastosis, and renal carcinoma (1, 5); and MC-29, a strain of avian leukemia virus that has been shown both by hybridization to DNA complementary to the *src* sequence (c[DNA]_{*src*}; ref. 5) and by nucleotide fingerprinting (6) to contain no *src*-specific sequences, transforms chicken embryo fibroblasts in culture (1). Additionally, normal cells appear to have *src*-specific sequences that are expressed during embryonic growth (5). In short, the mechanism of *src* gene action is far from clear.

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The isolation of active ingredients of croton oil by Hecker (7) has opened a whole new area of research, that of mechanism of action of tumor-promoting agents 8-11. It is now apparent that many characteristics induced by Rous sarcoma viruses (RSVs) are also induced by tumor promoters (10, 11). The overlap, however, is incomplete. Indeed, most of the effects of tumor promoters on normal cultured cells are analogous—qualitatively and quantitatively—to the effects of serum (12) or other growth factors and are less pronounced than those caused by viral transformation. In addition, some characteristics, such as transformed morphology (11) and growth in agar, are not induced by the promoters.

In this paper we show that at 41°C addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a potent chemical promoter, to chicken cells infected with LA24, a temperature-sensitive (ts) mutant of RSV with a defect in the *src* gene, produces a more exact phenocopy of transformed cells than does TPA treatment of normal cells. The product of the *src* gene at the nonpermissive temperature, therefore, appears to be either activated or replaced by the promoter. We propose that the product of the *src* gene itself may be a promoter rather than the initiator of transformation and that viral carcinogenesis may occur in two stages similar to those operating in mouse skin (13). The predictions of the model and the alternative interpretation of the data are discussed.

MATERIALS AND METHODS

Cell Culture and Virus Infection. Primary avian tendon (PAT) cells were prepared as described (14, 15). Chicken embryo fibroblasts (CEF) were prepared as described (16). The wild type (wt) and the ts mutant of Prague A-RSV (LA24) were focus purified (17) in our laboratory. The assay of focus-forming units indicated a virus concentration of 10⁷ transforming particles per ml of stock medium. Primary CEF cells were infected 1-4 hr after seeding with a multiplicity of infection of 0.1-0.5. Secondary cultures were prepared on day 5 after seeding, and cultures were kept at 39°C. In the case of LA24-infected cells, 24 hr after secondary seeding, cells were moved to either 41°C or 35°C incubators. Results reported in Tables 2 and 3 were obtained from secondary cultures. Other results (Fig. 2) were obtained from tertiary cultures prepared 2 days after secondary seeding (5 × 10⁵ cells per 50-mm dish) and shifted to the appropriate temperatures after 4 hr at 39°C. For experiments under agar, tertiary cells were prepared as above. A semisoft agar overlay (consisting of 100 ml of 2 × Dulbecco's modified Eagle's medium and F-10 in a 2:1 ratio, 50 ml of double-distilled H₂O, 50 ml of 1.8% agar, 11 ml of tryptose phosphate broth, 4.5% calf serum, 8 ml of 7.5% sodium bicarbonate, 2.3

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; RSV, Rous sarcoma virus; *src*, viral gene encoding the product(s) responsible for phenotypic transformation; PAT, primary avian tendon; CEF, chicken embryo fibroblasts; wt, wild type; ts, temperature-sensitive; 2-dGlc, 2-deoxy-D-glucose; dThd, thymidine; Me₂SO, dimethyl sulfoxide; PA, plasminogen activator.

ml of heated chicken serum, and 2.3 ml of 10% glucose) was added 4 hr after seeding. TPA was added 24–48 hr later in dimethyl sulfoxide (Me_2SO) that was diluted in F-12 or 199 medium. The appropriate amount of Me_2SO was added to control cultures. PAT cells were seeded in the absence of ascorbate as described above and grown at 39°C. Ascorbate has been shown to interfere with rapid spread of RNA virus infection in chicken cells (18) and was therefore eliminated during the early part of the transformation process in these experiments. Twenty-four hours after seeding, cells were infected with LA24 at a multiplicity of 1. Medium was changed daily. Cells were shifted to either 41°C or 35°C on day 5. Experiments were performed 2–3 days later.

Biochemical Assays. 2-Deoxy-D-glucose (2-dGlc) uptake and thymidine (dThd) incorporation were measured as described (16), using tritiated compounds. For collagen assay, cells were labeled with [^3H]proline at 50 $\mu\text{Ci/ml}$ (1 Ci = 3.7×10^{10} becquerels) for 3 hr and assayed as described (14, 15).

RESULTS

To observe enhancement in DNA synthesis and 2-dGlc uptake after TPA addition, the culture density and the growth rate had to be considered and controlled. While the rate of growth of RSV-transformed CEF or PAT cells is not different from that of uninfected cells during exponential growing phase, the rate of glucose uptake is higher (19–21) and the rate of collagen synthesis is lower (21, 22). We therefore set out to measure 2-dGlc uptake and collagen synthesis under the conditions in which normal cells, while at a reasonably high density so that the effect of TPA could be measured, were nevertheless still growing. The result with PAT cells indicated that addition of TPA to normal cells that were kept at 41°C resulted in an increase in 2-dGlc uptake as expected and additionally decreased the rate of collagen synthesis rapidly (Table 1). But the changes were smaller than those occurring after transformation (22). Addition of TPA to LA24-infected cells at 41°C, on the other hand, produced changes that were close to or even more drastic than those in LA24-infected cells grown at 35°C (Table 1). Sugar uptake was increased to the level of transformed cells by 5 hr and percent collagen synthesis dropped to 1/4.4 of its previous value (as opposed to 1/2.3 in normal cells) within 4–7 hr. The level of collagen synthesis in TPA-treated, LA24-infected cells at 41°C was thus comparable to or slightly lower than that in infected cells at the permissive temperature.

Table 1. Effect of TPA on sugar uptake and collagen synthesis in normal and infected PAT cells

Virus	Temp., °C	Addition	2-dGlc uptake, dpm/ μg protein per 5 min	Collagen synthesis	
				% [^3H]proline incorporated	Corrected % collagen
None	41	Me_2SO	205	33.1	8.6
None	41	TPA	445	17.1	3.8
ts	41	Me_2SO	220	22.8	5.3
ts	41	TPA	525	6.2	1.2
ts	35	Me_2SO	501	7.2	1.4
ts	35	TPA	565	7.1	1.4

Cells were seeded in 0.2% serum in F-12 medium. Seven days later TPA was added at 10 ng/ml of medium. 2-dGlc uptake was measured 5 hr later in a 5-min pulse. Counting efficiency was 33%. Collagen was assayed 4 hr later (a 3-hr pulse); the left column shows the percentage of [^3H]proline-containing material that is sensitive to purified collagenase; the right column is corrected for the fact that proline is 5.2 times more abundant in collagen than in other proteins. No ascorbic acid was present throughout the experiment. ts, LA24 RSV was used to infect cells. The values are the average of duplicate assays from duplicate plates of two experiments.

Perhaps the most striking finding was at the level of morphology. While normal PAT cells treated with TPA had a criss-cross pattern similar to that reported for CEF (11) (not shown), the LA24-infected PAT cells at 41°C assumed a morphology similar to fusiform transformed CEF (23) by 6 hr and looked entirely transformed by 10 hr (Fig. 1). The presence of TPA during a shift-down experiment pushed the transformation process further. Cells shifted in the presence of TPA for 12 hr were morphologically similar to cells that had been shifted to 35°C for 24–36 hr in the absence of TPA (data not shown).

Addition of TPA to normal CEF that were growing at a rate comparable to that of LA24-infected cells at the nonpermissive temperature (Exp. I, Table 2) increased the rate of sugar uptake by 30% in normal cells and 70% in infected cells. When the cells were at high density and were growing slowly, the increase in the rate of dThd incorporation into acid-precipitable material 24 hr after TPA addition was dramatic for both normal and LA24-infected cells at 41°C. Again, however, the increase in 2-dGlc uptake at 41°C was higher in the infected cells than in normal cells (Exp. II, Table 2).

In experiments in which TPA was added for 5 hr to normal and infected cells [both wt and (ts) RSV], the differential increase in 2-dGlc uptake was highest for cells infected with the

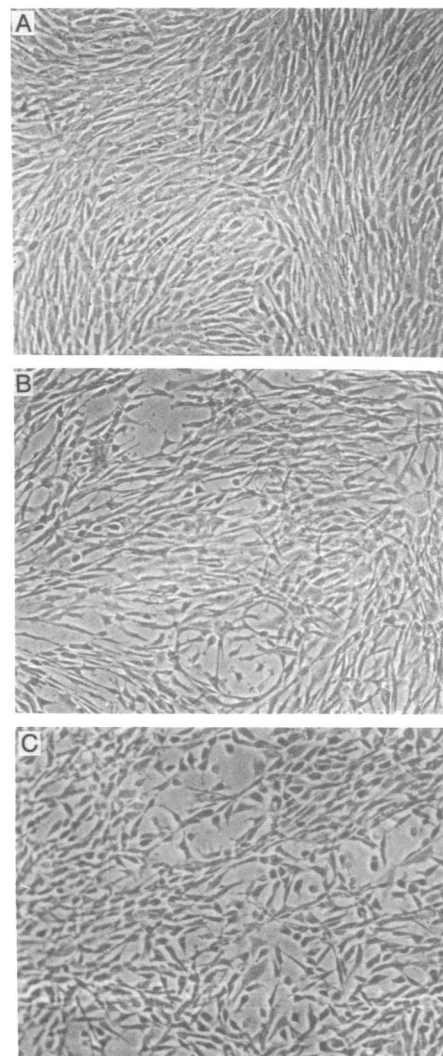


FIG. 1. Morphological effect of TPA on PAT cells. ($\times 80$.) PAT cells were seeded and infected with LA24. TPA (10 ng/ml) was added to infected cells at 41°C 7 days after initial seeding. (A) Me_2SO control after 6 hr; (B) TPA, 6 hr; (C) TPA, 10 hr.

Table 2. Effect of TPA on sugar uptake and DNA synthesis in normal and infected CEF at 41°C

Virus	Addition	Exp. I		Exp. II	
		2-dGlc uptake, dpm/ μ g prot. per 5 min	dThd incorp., dpm/ μ g prot. per hr	2-dGlc uptake, dpm/ μ g prot. per 5 min	dThd incorp., dpm/ μ g prot. per hr
None	Me ₂ SO	350	50	240	
None	TPA	405	670	880	
ts	Me ₂ SO	325	85	250	
ts	TPA	540	550	1290	

Secondary CEF were used. In Exp. I cells were seeded so that cultures were not too confluent and were still growing. TPA (10 ng/ml) was added 5 hr prior to assay. 2-[³H]dGlc concentration was 1 μ Ci/ml. Exp. II was performed on dense cultures and the assays were performed 24 hr after addition of TPA at 100 ng/ml. [³H]dThd and 2-[³H]dGlc were each at 2 μ Ci/ml. The values are the average of duplicate assays from duplicate plates.

mutant and kept at the nonpermissive temperature (Table 3). Cells infected with the wt RSV were least affected (this was true only when the cells were extremely well transformed). Normal cells and LA24-infected cells at 35°C were intermediates between the other two.

Attempts to produce foci with LA24-infected cells at 41°C in the presence of TPA have been unsuccessful so far, because the presence of TPA in a hard agar overlay for the length of time needed to produce visible foci (5–7 days) is toxic to the normal monolayer. Nevertheless, we did succeed in producing significant morphological alterations of LA24-infected CEF under semisoft agar after 24 hr of treatment at 41°C (Fig. 2). Normal cells under the same conditions showed only an increase in criss-cross patterns (Fig. 2). While dThd incorporation showed similar increases in TPA-treated normal and LA24-infected cells under semisoft agar, total protein content of infected, TPA-treated cultures increased more than that of normal, TPA-treated cells under agar (data not shown).

DISCUSSION

We have shown here that PAT cells and CEF cultures infected with a ts mutant of RSV with a defect in *src* gene function appear to become similar to transformed cells when they are treated with TPA at the nonpermissive temperature. It has previously been shown and is confirmed here that normal cells are also altered after TPA treatment, although by no means do they reach the transformed phenotype.

The morphological alterations brought about by the action of TPA on LA24-infected cells were dramatic, as shown in Figs. 1 and 2. It is difficult to quantitate morphological alterations; additionally, not only does the morphology of the transformed culture differ depending on the subgroup of RSV and the medium used, but the same virus causes two distinctive morpho-

Table 3. Percent increase in sugar uptake in normal and infected CEF after TPA addition

Virus	Temp., °C	% increase in 2-dGlc uptake	
		10 ng/ml	100 ng/ml
None	41	35	35
ts	41	52	55
wt	41	6	3
ts	35	23	13

Secondary cells were seeded and the indicated amount of TPA was added to each culture 48 hr later at the appropriate temperature. The rate of 2-dGlc uptake was measured 5 hr after addition of TPA. Percent increase is relative to Me₂SO controls. Results are the average of duplicate plates from two experiments.

logical changes depending on the nature of the host cell (1, 24). Nevertheless, morphology remains one of the better criteria of malignant transformation both in culture and *in vivo*. Only 10 hr after TPA treatment, under the same conditions in which TPA-treated normal cells were hardly changed, virus-infected PAT cells at 41°C looked radically altered (Fig. 1). Infected CEF cultures were not morphologically as responsive unless they were under semisoft agar (Fig. 2). CEF are regularly grown in much higher serum levels than PAT cells, which could affect some of the responses elicited after TPA addition.

The increase in the rate of 2-dGlc uptake is one of the accepted and commonly measured criteria of viral transformation. The increase is only quantitative and depends on the growth rate, medium conditions, cell shape, and density of the cultures (16, 19–21). Nevertheless, when all factors are controlled, sugar uptake is still higher in transformed cells. The 2-dGlc increases reported in this paper are not large, but the important point is that TPA-treated, infected cells at 41°C had a rate of sugar uptake that was higher than that of TPA-treated normal cells and approached or surpassed that of transformed cells at 35°C.

The drop in collagen synthesis, a differentiated function of tendon cells, after TPA treatment is reminiscent of the action of phorbol esters on terminal differentiation (25) and on the level of other differentiated gene products (26). Here again TPA and RNA tumor viruses shift the cells in the same direction although to differing degrees. In the PAT cell system, in which the level of collagen synthesis drops rapidly after viral transformation (22), addition of TPA also brings about a rapid decrease (Table 1). In CEF, in which collagen is synthesized at a low level and is not modulated easily by environmental factors (unpublished data), the decrease in collagen synthesis after TPA addition is reported to become significant only after prolonged treatment (one-fifth of its previous value after 5 days of treatment; P. M. Blumberg, personal communication). Thus different cell types respond to TPA or to the *src* gene product in characteristic manners.

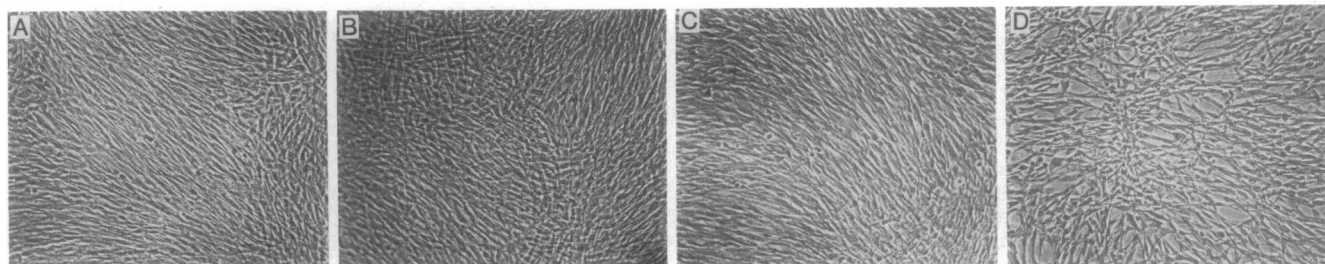


FIG. 2. Morphological effect of TPA on CEF under semisoft agar. ($\times 60$.) CEF cells were prepared and infected with LA24 ts RSV and kept at 40°C; 4 hr after tertiary seeding, an agar overlay was added. One day after addition of agar, TPA (10 ng/ml) was added under the agar with a syringe. Pictures were taken 24 hr later. (A) Uninfected cells, Me₂SO control; (B) uninfected, TPA; (C) ts-infected, Me₂SO; (D) ts-infected, TPA.

In comparing the action of promoters and that of RSV on uninfected CEF, Driedger and Blumberg (11) presented a model in which the pleiotropic effects of the two agents partially overlap. They suggested that other pharmacological agents in conjunction with TPA may generate a complete phenocopy of the transformed cells. We propose here that under appropriate conditions a complete overlap with transformed cells will occur if TPA is added to cells already infected with a tumor virus that has a defect in the *src* gene. Despite the preliminary nature of the results and the fact that there are other plausible explanations for the data (see below), the model that viral carcinogenesis may be a two- or multistep phenomenon and that the product of the *src* gene may be a promoter and thus secondary in the transformation process deserves consideration. It is consistent with the reversible nature of virus-induced "malignant transformation" as exemplified by transformation with ts mutants of RSV (27). The decay of the transforming activity in a shift-up experiment is, indeed, entirely analogous to the reversibility of the promoter activity. Furthermore, the model unifies the current concepts on the mechanism of malignant-transformation by viruses and carcinogens.

Predictions of the Model. (i) Addition of sufficient quantities of a promoter to cells infected with a ts mutant of RSV that has a mutation in the *src* gene should lead to a complete phenocopy of transformed cells at the nonpermissive temperature. The promoter may be phorbol esters, serum, single growth factors, hormones, metal ions (12), etc., alone or in combination. This prediction is fulfilled for TPA-treated LA24-infected cells by the criteria used in this paper. Results of experiments (10) in which plasminogen activator (PA) was measured after TPA addition to ts68-infected cells also fulfill this first prediction. CEF cells infected with ts68 at the nonpermissive temperature increased the level of PA production 6-fold upon addition of TPA. The level was even higher than that produced at the permissive temperature without TPA. Weinstein *et al.* (10) did not give an explanation for this remarkable synergism at 41°C. On the other hand, they did comment on the synergism that also occurs between TPA and the transformed cells at the permissive temperature. They pointed out that because this synergism appears to be multiplicative rather than additive, "the sarcoma genome and TPA act, at least in part, through different controlling elements" (10). In our hands, addition of TPA to well-transformed cells that were infected with wt RSV elevated the level of 2-dGlc uptake or decreased collagen synthesis only slightly, although there were additional changes in morphology. Addition of TPA to LA24-infected cells that were shifted to 35°C, however, caused significant acceleration in the transformation process (data not shown). This is to be expected if the hypothesis presented here is correct. In a shift-down experiment, time is needed for a buildup of the product(s) of the *src* gene, and an additional promoter would enhance the process appreciably. This may, in fact, partially explain the synergism of TPA and the activated product of *src* gene in PA production in ts68-infected cells at 36°C. Whatever the mechanism, the synergism of TPA and virus-infected cells at 41°C in terms of PA production is at least as significant as the synergism at 36°C. Thus, by a different criterion (PA production) and in a strain of virus (ts68) other than the one used in the present study, the first prediction of the model seems to be fulfilled.

(ii) The model predicts that transformation-defective viruses that have a deletion *within* the *src* gene should also produce a transformed phenocopy upon addition of a promoter. An easy assay for infection by these viruses is not available. The preliminary results of such experiments, while encouraging, are not reproducible as yet.

(iii) The product(s) of the *src* gene alone should be insufficient to cause transformation, although it should set into motion the events that are associated with growth promotion. In a recent study, McClain *et al.* (28) microinjected cytoplasmic extracts of cells transformed by RSV into normal cells and detected dissolution of microfilament bundles. We predict that microinjection of TPA-treated LA24-infected cells at 41°C will result in similar changes. Alternatively (and more likely), dissolution of microfilaments may be characteristic of growing cells as well as transformed cells. In fact we predict that addition of TPA to normal cells will also result in dissolution of the bundles.

(iv) Most importantly, the model predicts an initiation step that is linked to events prior to the activation of the *src* gene. Whether this event involves expression of another part of the viral genome [e.g., the "C" region (ref 1)] or whether it is entirely dependent on a specific integration site is a matter for further speculation.

Alternative Interpretation of the Data. (i) The LA24 virus used in these studies could be extremely "leaky." The *src* gene may be expressed partially at 41°C and the addition of the promoter somehow would bring about full expression. This possibility cannot be ruled out at this time. Previous studies with this particular clone of LA24 in our laboratory, however, have shown tight control at 41°C for morphology, 2-dGlc uptake, and collagen synthesis in PAT cells (22), and fluorescamine labeling, dThd incorporation, morphology, and 2-dGlc uptake in CEF (29).

(ii) The product of the *src* gene could be more than one protein. One may be expressed at 41°C and the other could be elaborated upon shift-down. The protein produced after shift-down could have a function similar to a promoter.

(iii) In all ts systems, the product of the *src* gene could be produced at both temperatures, but be inactive at the nonpermissive temperature. Addition of a promoter would then cause "activation" of the protein by some unknown mechanism. The rush of current literature on the nature of the product of the *src* gene (30-33) testifies to the fact that many other permutations of the above three alternatives are also possible. However, if one can "transform" a cell with a transformation-defective virus and promoters, all the alternative interpretations of the data become irrelevant. On the other hand, if upon addition of TPA the transformed phenotype can be generated with ts-infected cells only, the results discussed in this paper may still provide a means for deciphering the nature of the ts lesion of the *src* gene of RNA tumor viruses.

Some Aspects of the Literature Viewed in the Light of the Model. (i) Induction of leukemia (lymphatic as well as myeloid and erythroid) by viruses that do not contain the *src*-specific sequences but otherwise are very similar to nondefective RSV may be viewed as the result of "induction" (integration) without a need for promotion. Alternatively, barring the existence of a "leukemic gene" or recombination with a cellular *src* gene, promotion for blood-forming cells may be provided by other cellular and humoral factors *in vivo*.

(ii) Induction of carcinoma and fibrosarcoma *in vivo* and transformation of fibroblasts in culture by acute leukemia viruses such as MC29 and MH2 that also lack the *src* gene but are not identical with RSV in the rest of their genomes (6) would produce another gene product that would act as a promoter or induce a promoter-like activity in the cell.

(iii) Bishop, Stehelin, and coworkers (5, 34) have shown that *src*-specific sequences are endogenous to all normal avian species. They have further shown existence of RNA homologous to *src* in a variety of embryonic avian cells, indicating transcription from the *src* nucleotide sequences in normal avian

cells. They have proposed that *src* is part of the genome of normal cells, a part that can be mobilized during embryogenesis and growth (5). Indeed, there is now evidence that a 60,000 molecular weight protein that may be the same as the product of the *src* gene is also present in normal cells (J. M. Bishop, personal communication). The model presented here would support their proposal for the role of *src* in normal cells, although their interpretation of the reasons behind the transforming ability of *src* is different from ours.

(iv) The existence and the nature of a specific transformation gene in murine sarcoma viruses (MSVs) is inferred from the existence of conditional mutants even though the concept is not as well developed as in the case of avian sarcoma viruses. Nonproducing cells infected by certain ts mutants of Kirsten MSV, become wt for transformation if they are superinfected with a murine leukemia virus (MuLV) helper. Vogt states "If this effect represents true complementation, it would lead to the surprising conclusion that MuLV has a genetic function needed in the maintenance of MSV induced transformation" (1).

(v) The recent literature showing analogies between the product(s) of the *src* gene and epidermal growth factor in the murine system (35) and tumor promoters and epidermal growth factor in HeLa cells (36) taken together lend additional support to the "interchangeability" of the promoters and the product of the *src* gene.

(vi) There are numerous papers (which cannot all be cited here) such as the article by Jarrett *et al.* (37) and other articles (38, 39) that point to an intriguing synergism between chemicals and viruses in causing malignant tumors. Whether the virus is the "initiator" and the chemical the promoter or whether the chemical is the carcinogen and the virus the promoter remains to be determined in most of these cases. The model presented here predicts that a virus could play either or both roles. Additionally, the model provides for synergism between various promoters, the viral genome, and the host cell. The virus need not fulfill Koch's postulate entirely in order to be implicated as one of the causative agents of malignant tumors.

Finally, whether the predictions of the model are fulfilled or whether the data are the result of yet another twist in the expression of the *src* gene, we feel that a fresh look at current ideas and concepts of tumor virus carcinogenesis is in order. The elucidation of the mechanism by which tumor promoters exert their effects on normal and virus-infected cells should in any case aid in our understanding of both chemical and viral carcinogenesis. If the multistep model proves to be correct, the virus, perhaps, will still remain unique in that it can provide both initiation and promotion in rapidly integrated steps.

M.J.B. thanks the participants and organizers of the 1978 Gordon Research Conference on Cancer for creating an atmosphere that allowed these ideas to come to a focus, J. Michael Bishop and Peter Blumberg for sharing their unpublished results, Gordon Parry and Richard Schwarz for critical reading of the manuscript, and Steve Martin for the initial virus stock and also for listening. We thank Deborah Farson for excellent technical assistance and Beth Klingel for typing the manuscript. This investigation was funded in part by the National Science Foundation (Grant PCM 77-14982) and in part by the Division of Biomedical and Environmental Research of the Department of Energy.

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