Title
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Publication Date
1978-10-01

Submitted to Proceedings of the National
Academy of Sciences of the USA

LBL-8370 c.2
Preprint

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October 1978

Prepared for the U. S. Department of Energy
under Contract W-7405-ENG-48

DISCLAIMER

IS THE PRODUCT OF THE *Src* GENOME A PROMOTER?

Key words: Rous sarcoma virus, phorbol esters, chick cells, collagen, viral carcinogenesis.

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Classification: Cell Biology

Running Title: The *Src* gene product may be a promoter

ABBREVIATIONS USED

TPA, 12-O-tetradecanoyl-phorbol-13-acetate; RSV, Rous sarcoma virus; PAT, primary avian tendon; CEF, chick embryo fibroblasts; *wt* and *ts* RSV, wild type and temperature-sensitive mutant of RSV; 2-dg, 2-deoxy-D-glucose; Tdr, thymidine; DMSO, dimethyl sulfoxide; PA, plasminogen activator.

ABSTRACT

Addition of a potent promotor 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to primary avian tendon or chick embryo fibroblast cells infected with a temperature sensitive mutant of Rous sarcoma virus produced a complete transformed phenocopy at the non-permissive 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 alterations 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 integration of all or part of the viral genome and a promotion step (itself a multi-step process) caused by the activation of the *src* gene. The latter could be enhanced or replaced by other promoting agents.

INTRODUCTION

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 identify 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 have been turned around recently and are used to support the argument that viral transformation is not á 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, even in the most straightforward and widely used system, that of chick embryo fibroblasts transformed by Rous sarcoma viruses, many puzzles remain. For example, if indeed *src* is the "oncogene", its deletion or its absence from a viral genome should make the virus incapable of causing malignancy in vivo or transformation in culture. Yet many "*src*-defective" viruses such as RAV-2, RAV-6, RAV-50, and AMV induce a variety of tumors in vivo, including

lymphoid leukosis, myelobalstosis, and renal carcinoma (1,5); and MC-29, a strain of avian leukosis virus that has been shown both by hybridization to DNA complementary to the *src* sequence (c[DNA]$_{src}$; 5) and by nucleotide finger printing (6) to contain no *src*-specific sequences, transforms chick embryo fibroblasts in culture (1,7). In addition, normal cells appear to have *src*-specific sequences which are expressed during embryonic growth (5).

The isolation of active ingredients of croton oil by Hecker (8,9) has set into motion a whole new area of research, that of mechanism of action of tumor promoting agents. Cell culture is being used extensively and chick embryo fibroblasts in particular have become the model of choice (10-13). It is now apparent that many parameters induced by Rous sarcoma viruses are also induced by tumor promoters (11,13). The overlap, however, is incomplete. Indeed, most of the action of tumor promotors on normal cultured cells are analogous-qualitatively and quantitatively-to that induced by serum (14) or other growth factors and are less pronounced than those caused by viral-transformation. In addition, some parameters such as transformed morphology (13) and growth in agar are not induced by the promoters. Weinstein and coworkers have also indicated that another difference may lie in the reversibility of promoter action, i.e., while the TPA-induced effects are reversible, that of viral transformation is not (12). The reversibility argument, however, cannot be sustained. The very existence of RSV mutants which are temperature sensitive for transformation (15) indicates that the virus-induced transformation phenotype is at least as reversible as the promoter-induced phenotype.

In this paper we show that at 41° addition of TPA, a potent chemical promoter, to chick cells infected with LA24, a temperature sensitive mutant of RSV with a defect in *src* gene, reproduces a more exact phenocopy of

transformed cells than normal, TPA-treated cells. The product of the *src* gene at the non-permissive temperature, therefore, appears to be either activated or replaced by the promoter. We propose that the latter may be the case, i.e., that the product of the *src* genome itself may be a promoter rather than the initiator of transformation. In trying to reproduce the transformed phenotype completely, the promoter should be added not to an uninfected cell, but to one which has been already "initiated"--in this case by virus infection and integration. The predictions of the model and the alternative interpretation of the data are discussed.

MATERIALS AND METHODS

Cell culture. Primary avian tendon (PAT) cells were prepared as described previously (16,17). Tendon cells from 16-day old chick embryos were dissociated into single cells and .5-.8 x 10^6 cells were allowed to attach in 50 mm culture dishes (Falcon) in medium F12 for one h. The medium was then replaced and changed daily with 0.15%-0.2% fetal calf serum (Gibco, Grand Island, NY; deactivated for 30 min at 56°C) with or without 50 µg/ml ascorbic acid. Chick embryo fibroblast (CEF) were prepared as described (18).

Virus infection and transformation. The wild type and the temperature-sensitive mutant of Prague A-RSV (LA24) were provided by Dr. Steven Martin, Zoology Dept., UC Berkeley. The viruses were focus purified further in our laboratory. The assay of focus forming units (19) indicated a virus concentration of 10^7 transforming particles per ml of stock medium. Primary CEF cells were infected 1-4 hours after seeding with a multiplicity of infection of .1-.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 hrs after secondary seeding cells were moved either to 41° or 35° incubators.

Results reported in Tables 2 and 3 were done on secondary cultures. Other results (Fig. 2) were done on tertiary cultures prepared two days after secondary seeding (5 x 10^5 cells per 50 mm dish) and shifted to the appropriate temperatures after 4 hrs at 39°. For experiments under agar, tertiary cells were prepared as above. A semisoft agar overlay (consisting of 100 ml 2 x DME and F-10 in a 2:1 ratio, 50 ml DDH_{20}, 50 ml 1.8% agar, 11 ml tryptose phosphate broth, 4.5% calf serum, 8 ml 7.5% bicarbonate, 2.3 ml heated chick serum, 2.3 ml 10% glucose solution) was added 4 hrs after seeding. TPA was added 24-48 hrs later in DMSO which was diluted in medium F-12 or 199. The appropriate amount of DMSO was added to control cultures. PAT cells were seeded in the absence of ascorbate as described above and grown at 39°. Ascorbate has been shown to interfere with rapid spread of RNA-virus infection in chick cells (20) and was thus eliminated in the early part of the transformation process in these experiments. Twenty-four hrs after seeding, cells were infected with LA24 at a multiplicity of 1. Medium was changed daily. Cells were shifted to either 41° or 35° on day 5. Experiments were performed 2-3 days later.

Biochemical assays. 2-Deoxy-D-glucose (2-dg) uptake and thymidine (Tdr) incoration were performed as described previously (18) using tritiated compounds obtained from New England Nuclear. For collagen assay, cells were labeled with 50 μCi/ml [^{3}H]-proline (New England Nuclear) for 3 hrs and assayed as described (16).

RESULTS

A search of the literature indicated that the addition of TPA to normal cells produced changes which in most instances were similar to serum and other growth promoting factors (13,14); i.e., a normal cell plus promoter is more analogous to a rapidly growing cell than to a malignant tumor.

While the rate of growth of Rous-transformed CEF or PAT cells in not different from that of uninfected cells during exponential growing phase, their rate of glucose uptake is higher (18,21) and that of collagen synthesis is lower (23,24). We therefore set out to measure 2-dg uptake and collagen synthesis under the conditions where 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 which were kept at 41° resulted in an increase in 2-dg uptake as expected and additionally decreased the rate of collagen synthesis rapidly (Table 1). But the changes were smaller than those occurring after transformation (23). Addition of TPA to LA24-infected cells at 41°, on the other hand, produced changes that were close or even more drastic than those in LA24-infected cells grown at 35° (Table 1). Sugar uptake was increased to the level of transformed cells by 5 hrs and percent collagen synthesis was dropped 4.4-fold (as opposed to 2.3-fold in normal cells) within 4-7 hrs. The level of collagen synthesis in TPA-treated, LA24-infected cells at 41° was thus comparable or slightly lower than infected cells at the permissive temperature.

Perhaps the most striking finding was at the level of morphology. While normal PAT cells treated with TPA had a slight criss-cross pattern similar to that reported for CEF (13) (not shown), the LA24-infected PAT cells at 41° assumed a morphology which was similar to fusiform transformed CEF (25) by 6 hrs and looked entirely transformed and distinctly different from TPA-treated normal cells by 10 hrs (Fig. 1). The presence of TPA during a shift-down experiment pushed the transformation processs further. Cells shifted in the presence of TPA for 12 hrs were morphologically similar to cells which had been shifted to 35° for 24-36 hrs in the absence of TPA (data not shown).

The effect of TPA on LA24-infected CEF

Addition of TPA to normal chick embryo fibroblasts which were growing at a rate comparable to LA24-infected cells at the non-permissive 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 thymidine incorporation into acid-precipitable material 24 hr after TPA addition was dramatic for both normal and LA24-infected cells at 41°. Again, however, the increase in 2-dg uptake at 41° was higher in the infected cells than in normal cells (Exp. II, Table 2).

In experiments where TPA was added for 5 hrs to normal and infected cells (both wild type and LA24 RSV), the differential increase in 2-dg uptake was highest for cells infected with the mutant and kept at the non-permissive 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° were intermediates between the other two.

Attempts to produce foci with LA24-infected cells at 41° in the presence of TPA has been unsuccessful so far. But this perhaps can be explained: the presence of TPA in the hard agar overlay for the length of time needed to have visible foci (5-7 days) was toxic to the normal monolayer. Experiments are in progress to overcome this problem. Nevertheless, we did succeed in producing significant morphological alterations of LA24-infected CEF under semi-soft agar after 24 hrs of treatment (Fig. 2; see Materials and Methods). Normal cells under the same conditions showed only a slight increase in criss-cross patterns (Fig. 2). While thymidine incorporation showed similar increases in TPA-treated normal and LA24-infected cells under semi-soft 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 temperature sensitive 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 non-permissive temperature. While an impressive and puzzling synergism between the active *src* gene product (ts 68-infected CEF at 36°) and TPA in elaboration of plasminogen activator had been observed and commented on (12), the general synergism between TPA and virus-infected (but "untransformed") cells at 41° had not attracted much attention.

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. In comparing the action of promoters and that of RSV on uninfected CEF, Driedger and Blumberg (13) presented a model where the pleiotropic effects of the two agents partially overlap. They suggested that other pharmocological 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 which has a defect in the *src* gene.

The morphological alterations brought about by the action of TPA on LA24-infected cells was dramatic as shown in Figs. 1 and 2. It is difficult to quantitate morphological alterations; additionally not only the morphology of the transformed culture differs depending on the subgroup of RSV and the medium used, but the same virus causes two distinctive morphological changes depending on the nature of the host cell (1,26). Nevertheless, morphology still remains one of the better criteria of malignant transformation especially for primary cultures (as opposed to cell lines). Only 10 hrs after TPA treatment, under the same conditions where TPA-treated normal cells were

hardly changed, virus infected PAT cells at 41° looked radically altered (Fig. 1). 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 (see Methods) which could affect some of the responses elicited after TPA addition. In reproducing the transformed phenotype, there is no reason to expect that TPA treated, virus-infected cells should look exactly similar to cells infected with wild type RSV. If the activation of the *src* gene basically provides a promoter-like activity, other growth promoting agents--serum, single growth factors, hormones, metal ions (14), etc. alone or in combination would be expected to also mimic the transformation process and cause a variety of morphological and biochemical alterations when added to untransformed, infected cells.

The increase in the rate of 2-dg 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 (18,21-23). Nevertheless, when all factors are controlled, sugar uptake is still higher in transformed cells. The 2-dg increases reported in this paper are not large, but the important point is that TPA-treated, infected cells at 41° had a rate of sugar uptake which was higher than TPA-treated normal cells and approached or surpassed that of transformed cells at 35°.

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 (27,11), and on the level of other differentiated gene products (28). Here again TPA and RNA-tumor viruses shift the cells in the same direction although to differing degrees. In the PAT cell system where the level of collagen synthesis drops rapidly after viral transformation (24), addition of TPA also brings about a rapid decrease

(Table 1). In CEF where collagen is synthesized at a low rate and is not modulated easily by environmental factors (unpublished data), the decrease in collagen synthesis after TPA addition is reported to be significant but very slow (a 5-fold reduction after 5 days of treatment; 29). Thus, different cell types respond to TPA or to the *src* gene product in a manner characteristic for that cell system.

Despite the preliminary nature of the results and the fact that there are other plausible explanations for the data (see below), the hypothesis that viral-carcinogenesis may be a two- or multi-step phenomena and that the activation of the *src* gene may not be the crucial step deserves some consideration. To begin with it explains the reversibile nature of virus-induced "malignant-transformation" as exemplified by transformation with temperature-sensitive mutants of RSV. The decay of the transforming activity in a shift-up experiment is, indeed, entirely analogous to the reversibility of the promoter activity. Furthermore, the hypothesis unifies the current concepts on the mechanism of malignant-transformation by viruses and carcinogens. The model makes a number of important predictions which are discussed below.

A. Predictions of the Model

1. Addition of any agent with promotor activity to cells infected with a temperature sensitive mutant of RSV which has a mutation in the *src* genome should lead to a complete phenocopy of transformed cells at the non-permissive temperature. This prediction is fulfilled for LA24-infected cells by the criteria used in this paper as discussed above. A piece of data in the literature where plasminogen activator (PA) was measured after TPA addition to ts68-infected cells also supports this first prediction. Weinstein et al. (12) reported that CEF cells, infected with ts68 at the non-permissive temperature had a level of PA which produced 5% fibrinolysis at a given time. At the permissive temperature the cells produced 23%,

an increase of 4.6-fold. Addition of 30 ng/ml TPA to ts 68-infected cells at 41° increased the plasminogen activator level 6 folds so that the fibrinolysis reached 30%, even higher than transformed cells at the permissive temperature. Weinstein et al. do not give an explanation for this remarkable synergism at 41° where infected cells are supposed to act like normal cells. They comment, however, on the synergism that also occurs between TPA and the transformed cells at 35° (from 23 to 65% in this experiment). They point out that since this synergism appears to be multiplicative rather than additive, therefore, "the sarcoma genome and TPA act, at least in part, through different controlling elements" (12). In our hands, addition of TPA to well-transformed cells which were infected with wt-RSV elevated the level of 2-dg uptake or decreased collagen synthesis only slightly, although there were additional changes in morphology (which may be related to the sharp increase in PA observed by Weinstein et al.). Addition of TPA to LA24-infected cells which were shifted to 35°, 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 *src* genome and an additional promoter would enhance the process. This may, in fact, partially explain the synergism of TPA and the activated *src* genome in PA production in ts68-infected cells at 36°. Whatever the mechanism, the significance of the finding that TPA-treated cells infected with a *ts*-mutant at non-permissive temperature resemble the cells at the permissive temperature in terms of PA production should not be overlooked. Thus, by a different criteria (PA production) and in a strain of virus other than the one used in the present study (ts68) the first prediction of the model seems to be fulfilled.

There is a very important correlary to this first point: The model would predict that transformation-defective viruses which have a deletion

within the *src* genome should also produce a transformed phenocopy upon addition of a promoter (see Part B). These experiments are in progress.

2. Transfection experiments with $c[DNA]_{src}$ should be completely successful only when the cells are already infected with transformation-defective viruses, although the integration site of *src*-specific sequences itself could provide the "initiation" step.

3. The product of the *src* genome alone should be insufficient to cause transformation, although it could set into motion the events that lead to growth. In a recent study, McClain et al. (30) microinjected cytoplasmic extracts of cells transformed by RSV into normal cells and detected dissolution of microfilament bundles. We would predict that microinjection of TPA-treated LA24-infected cells at 41° would result in similar changes. It is possible, however, that dissolution of microfilaments is characteristic of growing cells as well as transformed cells in which case addition of promoters to normal cells should lead to the same phenomenon. The demonstration that *src* sequences are present in normal chick cells (31) and that *src*-specific RNA is made during embryogenesis (5) may be taken to mean that *src's* presence or its expression is not sufficient for cancer induction. The hypothesis by Bishop et al. (5) that in avian cells *src* is involved in cell growth would be supported by our model (see below).

4. Most importantly, the model predicts an initiation step which is linked not to the activation of the *src* gene but to integration of some part of the virus. Whether this event also involves expression of another part of the viral genome or whether it is entirely dependent on a specific integration site is a matter for further speculation.

B. Alternative Interpretation of the Data

1. The LA24 virus used in these studies could be extremely "leaky". The *src* gene may be expressed partially at 41° and the addition of the promoter somehow would bring about full expression. This possibility cannot be ruled out at this time. However, there is extensive literature on biochemical characterization of CEF cells infected with LA24 RSV at both permissive and non-permissive temperatures in culture and there is no reason to believe that these viruses are any more "leaky" than other *ts* mutants. Indeed, they appear to be less so. Previous studies with this particular clone of LA24 in our laboratory has shown tight control at 41° for morphology, 2-dg uptake and collagen synthesis in PAT cells (24), and fluorescamine labeling, Tdr incorporation, morphology and 2-dg uptake in CEF (32). Additionally, as cited above, in at least another laboratory and with an entirely different *ts* virus (ts68), when TPA was present, cells infected at the non-permissive temperature became completely similar to cells infected at the permissive temperature in terms of PA production (12).

2. The product of the *src* genome could be more than one protein. One may be expressed at 41° and the other could be elaborated upon shift-down. The latter could have a function similar to a promoter. This is entirely consistent with the data and would still suggest a promoter-like activity for a gene product.

3. In all *ts* systems, the product of the *src* gene could be produced at both temperatures, but be inactive at the non-permissive temperature. Addition of a promoter would then cause "activation" of the protein by some unknown mechanism. Brugge and Erikson (33) have identified a 60,000 dalton transformation-specific antigen and Collett and Erikson (34) have shown that it contains protein kinase activity. The expression of kinase activity was shown to be temperature-dependent in cells infected with *ts*-mutants of RSV. Jay et al. have reported that an antisera obtained from rats bearing RSV tumors, cross reacts with a 56,000 dalton protein at both permissive and non-permissive temperatures but that the protein is absent in cells infected with a transformed-defected virus. It is not clear whether the 56,000 MW protein is the same as the one described by Collett and Erikson. Nevertheless, one can postulate that the kinase itself or one of the steps leading to the final transformation event could be activated at the non-permissive temperature by TPA.

There are other permutations of the above 3 alternatives which would also be consistent with the data. However, we still prefer the initial model because it provides some explanation for other data in the literature (see below). If one can "transform" a cell with a transformation-defective virus and promoters, all the other alternative interpretations of the data would become irrevelent. On the other hand, if upon addition of TPA the transformed phenotype can be generated with *ts*-infected cells only, the promoters may provide a new means for deciphering the nature of the temperature-sensitive lesion of the *src* gene of RNA-tumor viruses.

C. Some Aspects of the Literature Viewed in the Light of the Model

1. Induction of leukemia (lymphatic as well as myeloid and erythroid) by viruses which do not contain the *src*-specific sequences but otherwise are very similar to non-defective RSV may be viewed as the result of

"induction" (integration) without a need for promotion. Alternatively, barring the existence of a "leukemic gene", promotion for blood-forming cells may be provided by other cellular and humoral factors in vivo.

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

3. Bishop, Stehelin and coworkers have shown that *src*-specific sequences are endogeneous to all normal avian species (5,24). 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 which can be mobilized during embryogenesis and growth (5). The model presented here is entirely in keeping with their proposal. As mentioned above, the product of the *src* gene could act as a promoter essentially as other growth promoting agents do. Its presence alone would lead to growth and all its manifestations without the malignant end point. Indeed, there is now evidence that a 60,000 MW protein which may be the same as the product of the *src* genome is also present in normal cells (36).

4. The existence and the nature of a specific transformation gene in murine sarcoma viruses (MSV) 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. Non-producing 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).

There are numerous recent literature which cannot all be cited in this paper such as the article by Jarrett et al. and others (37-39) which 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 would predict 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 is 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 multi-step 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.

ACKNOWLEDGEMENTS

MJB would like to thank the participants and organizers of the 1978 Cancer Gordon Research Conference for creating an atmosphere which allowed these ideas to come to a focus, J. Michael Bishop and Peter Blumberg for sharing their unpublished resuts, Gordon Parry for critical reading of the manuscript, and Steve Martin for the initial virus stock and also for listening and suggesting that the "alternative" was a much more plausible explanation than the model! 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 No. PCM 77-14982) and in part by the Division of Biomedical and Environmental Research of the Department of Energy.

REFERENCES

1. Vogt, P.K. (1977) in Comprehensive Virology, eds. Fraenkel-Conrat, H. & Wagner, R. (Plenum Press, New York), Vol. 9, pp. 341-455.

2. Hanafusa, H. (1977) in Comprehensive Virology, eds. Fraenkel-Conrat, H. & Wagner, R. (Plenum Press, New York), Vol 10, pp. 401-483.

3. Tooze, J. ed. (1973) The Molecular Biology of Tumor Viruses (Cold Spring Harbor Laboratory, New York).

4. Baltimore, D. (1975) Tumor Viruses: 1974. Cold Springs Harbor Symp. Quant. Biol. 39, 1187.

5. Bishop, J.M., Stehelin, D., Tal, J., Fujita, D., Spector, D., Roulland-Dussoix, T., Padgett, T. & Varmus, H.E. (1977) The Molecular Biology of the Mammalian Genetic Apparatus, ed. Ts'o, P. (Elsevier/North Holland Biomedical Press), pp. 277-287.

6. Duesberg, P.H., Bister, K. & Vogt, P. (1977) Proc. Nat. Acad. Sci. USA USA 74, 4320.

7. Langlois, A.J. & Beard, J.W. (1967) Proc. Soc. Exp. Biol. Med. 126, 718.

8. Hecker, E. (1967) Naturwissenschaften 54, 282.

9. Hecker, E. (1971) in Methods in Cancer Research, ed. Busch, H. (Academic Press, N.Y.), 439-484.

10. Diamond, L., O'Brian, S., Donaldson, C. & Shinizu, Y. (1974) Int. J. Cancer 13, 721.

11. Weinstein, I.B. & Wigler, M. (1977) Nature 270, 659.

12. Weinstein, I.B., Wigler, M. & Pietropaolo, C. (1977) in The Origins of Human Cancer (Cold Spring Harbor Laboratory, New York) 751-772.

13. Driedger, P.E. & Blumberg, P.M. (1977) Cancer Res. 37, 3257.

14. Rubin, H. & Koide, T. (1975) J. Cell Physiol. 86, 47.

15. Martin, G.S. (1970) Nature 227, 1021.

16. Schwarz, R.I., Colarusso, L. & Doty, P. (1976) Exp. Cell Res. 102, 63.

17. Schwarz, R.I. & Bissell, M.J. (1977) Proc. Natl. Acad. Sci. USA 74, 4453.

18. Bissell, M.J., Farson, D. & Tung, A.S.-C. (1977) J. Supramolecular Struc. 6, 1.

19. Rubin, H. (1960) Virology 10, 29.

20. Bissell, M.J., Hatié, C., Farson, D., Schwarz, R.I. & Soo, W.J. (1978) J. Cell. Biol. 79 (Part 1), 401a.

21. Dolberg, D., Bassham, J.A. & Bissell, M.J. (1975) Exp. Cell Res. 96, 129.

22. Weber, M.J. (1973) J. Biol. Chem. 248, 2978.

23. Schwarz, R.I., Farson, D.A., Soo, W.-J. & Bissell, M.J. (1978) J. Cell Biol. In press.

24. Soo, W.-J., Schwarz, R.I., Bassham, J.A. & Bissell, M.J. Submitted for publication.

25. Martin, G.S. (1971) in The Biology of Oncogenic Viruses, ed. Silvestri, L.G. (North-Holland, Amsterdam), pp. 370-325.

26. Gaff,T. & Friis, R.R. (1973) Virology 56, 369.

27. Diamond, L., O'Brien, T. & Rivera, G (1978) in Carcinogenesis Vol. 2 Mechanisms of Tumor Promotion and Carcinogenesis, eds. Sluger, T.J., Sinak, A, & Boutwell, R.K. (Raven Press, New York), pp. 335-341.

28. Pacifici, M & Holtzer, H. (1977) Am. J. Anat. 150, 207.

29. Blumberg, Peter (personal communication).

30. McClain, D.A., Maness, P.F. & Edelman, G.M. (1978) Proc. Natl. Acad. Sci. USA 75, 2750.

31. Stehelin, D., Gumtaka, R.V., Varmus, H.E. & Bishop, J.M. (1976) J. Mol. Biol. 101, 349.

32. Parry, G. & Hawkes, S.P. (1978) Proc. Natl. Acad. Sci. USA 75, 3703.

33. Brugge, J.S. & Erikson, R.L. (1977) Nature 269, 346.

34. Collett, M.S. & Erikson, R.L. (1978) Proc. Natl. Acad. Sci. USA, 75, 2021.

35. Jay, G., Shiu, R.P.C., Jay, R.T., Levine, A.S. & Pastan, I. (1978) Cell 13, 527.

36. Bishop, J. Michael (personal communication).

37. Jarrett, W.F.H., McNeil, P.E., Grimshaw, W.T.R., Selman, J.E. and McIntyre, W.I.M. (1978) Nature 274, 215.

38. Calvin, M. (1975) Die Naturwissen-schaften 62, 405.

39. Stitch, H.F., Kieser, D. Laishes, B.A., San, R.H.C. and Warren, P. (1975) Gann Monograph on Cancer Res. 17, 3.

TABLE 1

Effect of TPA on Sugar Uptake and Collagen Synthesis in PAT Cells

Cell Type	2-dg (DPM/ µg Prot./5 min)	Collagen Synthesis	
		% Proline Incorporated	Corrected % Collagen
$N_{41°}$ + DMSO	205	33.1	8.6
$N_{41°}$ + TPA	445	17.1	3.8
$TS_{41°}$ + DMSo	220	22.80	5.3
$TS_{41°}$ + TPA	525	6.2	1.2
$TS_{35°}$ + DMSO	501	7.2	1.4
$TS_{35°}$ + TPA	565	7.1	1.4

Cells were seeded in 0.2% serum in medium F-12 and treated as described in Materials and Methods. Seven days later TPA was added at 10 ng/ml of medium. 2-dg was measured 5 hrs later in a 5 min. pulse. Collagen assay was performed 4 hrs later (a 3 hr pulse; see Methods): the left column shows the percentage of [^{3}H]-proline incorporated counts which is sensitive to purified collagenase; the right column is the same valve corrected for the fact that proline appears 5.2 times more in collagen than in other proteins. No ascorbic acid was present throughout the experiment for reasons described in the Methods. In the presence of ascorbate, the level of collagen synthesis would be at least twice as high in normal cells at this density (17) while the level will not modulate in well transformed cells (23). The rate of collagen synthesis is extremely sensitive to environmental perturbation in PAT cells.

The lower level of collagen synthesis in infected cells at 41° as compared to

normal control in these experiments is due to a slight lowering of the temperature that occurred during manipulations. In other experiments where the same parameter was measured at 41° in LA24-infected cells and the temperature was rigorously controlled, it was strictly comparable to normal controls (24). N, normal cells; TS, cells infected with LA24 RSV and grown at the indicated temperatures. The values are the average of duplicate assays from duplicate plates of 2 experiments.

TABLE 2

Effect of TPA on Sugar Uptake and DNA Synthesis in CEF

Cell Type	Exp. I	Exp. II	
	2-dg, DPM/µg Prot/5 min	Tdr, DPM/µg Prot/ 1 h	2-dg, DPM/µg Prot/5 min
$N_{41°}$ + DMSO	350	50	240
$N_{41°}$ + TPA	405	670	880
$TS_{41°}$ + DMSO	325	85	250
$TS_{41°}$ + TPA	540	550	1290

Secondary CEF were grown as described in Materials and Methods. In the first experiment 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. [^{3}H]-2-dg concentration was 1 µci/ml. The second experiment was performed on dense cultures and the assays were performed 24 hrs after addition of 100 ng/ml TPA[^{3}H]-Tdr and [^{3}H]-2-dg were at 2 µci/ml each. The values are the average of duplicate assays from duplicate plates.

TABLE 3

Percent Increase in Sugar Uptake in CEF After TPA Addition

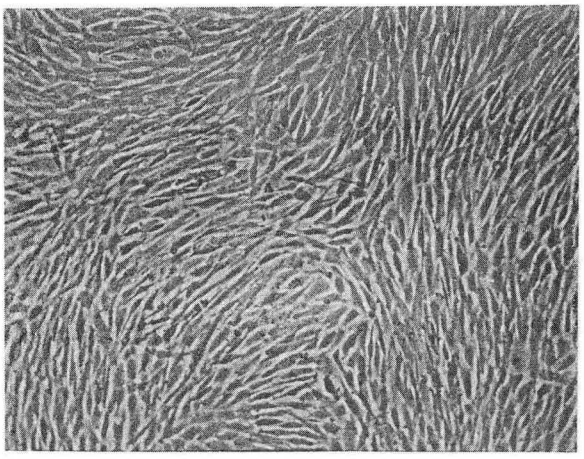
TPA added ng/ml	$N_{41°}$	$TS_{41°}$	$WT_{41°}$	$TS_{35°}$
	% increase over DMSO controls after 5 hrs.			
10	35	52	6	23
100	35	55	3	13

Secondary cells were seeded as described in Materials and Methods. The indicated amount of TPA was added to each culture 48 hrs later at the approproate temperature. The rate of 2-dg uptake was measured 5 hrs after addition of TPA. Percent increase was calculated as [(CPM/μg Prot. in treated sample - CPM/μg Prot. in DMSO control)/CPM/μg Prot. in DMSO control] x 100. Results are the average of duplicate plates from 2 experiments.

FIGURE LEGENDS

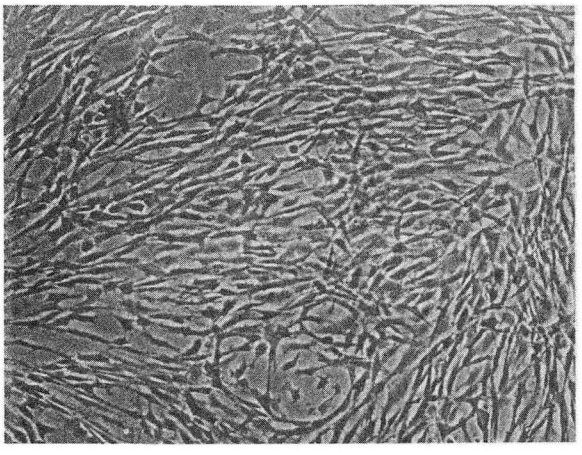
Fig. 1. Morphological effect of TPA on PAT cells. PAT cells were seeded and infected with LA24 as described in Materials and Methods. TPA (10 ng/ml) was added 7 days after initial seeding to infected cells at 41°. Pictures were taken 6 and 10 hrs later.

Fig. 2. Morphological effect of TPA on CEF under semisoft agar. CEF cells were prepared and infected with LA24 as described im Materials and Methods. Four hrs after tertiary seeding, an agar overlay was added as described. One day after addition of agar, TPA (10 ng/ml) was added under the agar. Pictures were taken 24 hrs later.



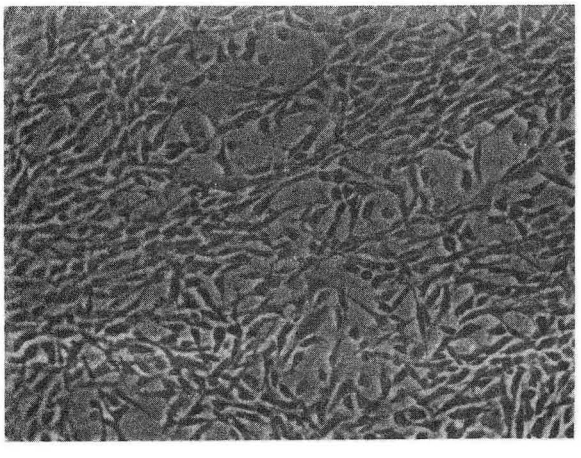
TS_{41} + DMSO

6 hours



TS_{41} + TPA

6 hours



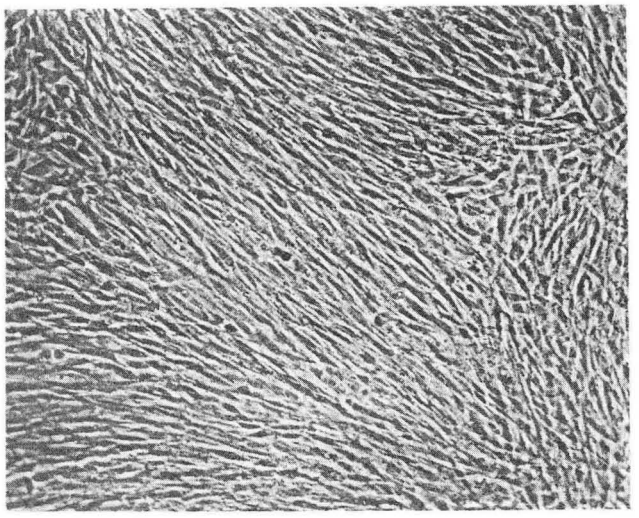
TS_{41} + TPA

10 hours

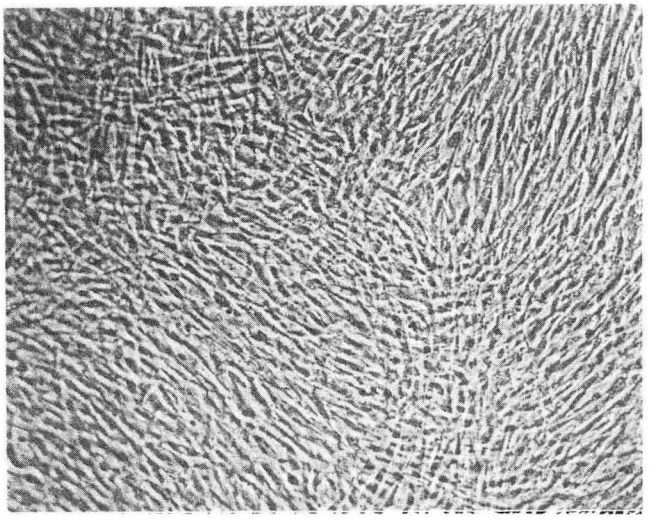
100 μm

XBB 7810-13064

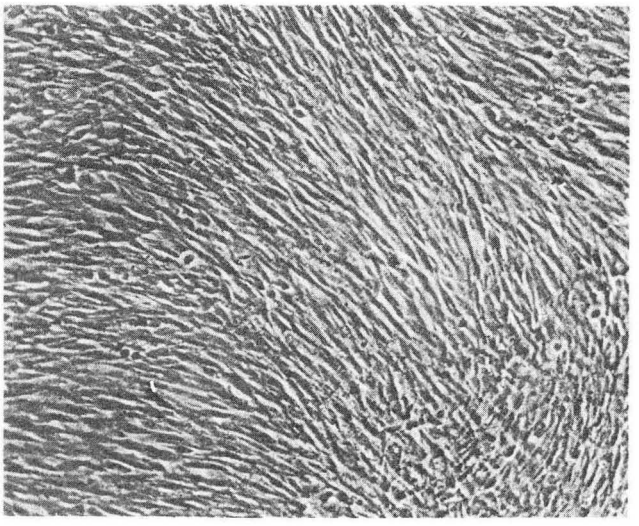
Fig. 1



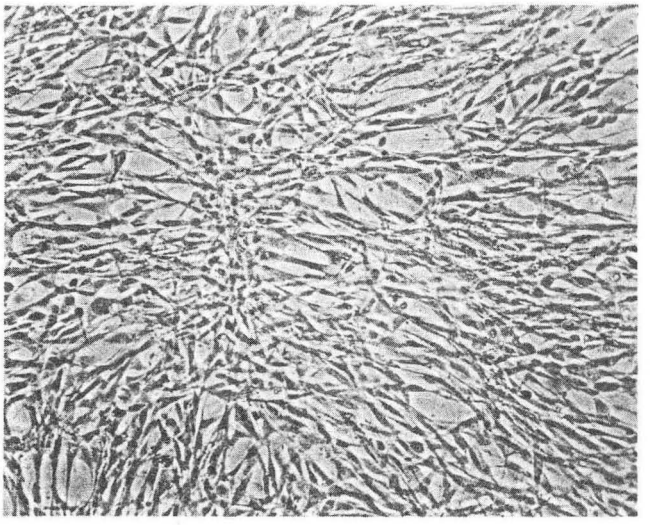
N_{41} + DMSO



N_{41} + TPA



TS_{41} + DMSO



TS_{41} + TPA

100μm

XBB 7810-13065

Fig. 2

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