

Polyomavirus Middle Tumor Antigen Increases Responsiveness to Growth Factors

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The middle tumor antigen (mT) of polyomavirus is unable to transform a clone of NIH 3T3 cells to anchorage independence (L. Raptis and J. B. Bolen, *J. Virol.* 63:753–758, 1989). However, this oncogene increased the responsiveness of these cells to the growth factors (α -like and β -type transforming growth factors) produced by cells possessing the whole transforming region of polyomavirus. This resulted in the growth of NIH 3T3 cells, expressing mT under control of the dexamethasone-regulatable mouse mammary tumor virus promoter, in agar medium supplemented with these growth factors upon addition of the inducer. Therefore, mT, a transforming oncogene, is able to enhance the responsiveness of established cells to growth factors, a property previously attributed primarily to *myc* and other establishment type oncogenes.

Oncogenes can be classified into two broad functional groups. The first group consists of nuclear proteins, which are only able to “immortalize,” or establish, primary cells in culture, i.e., lock these cells in a proliferative mode (11, 26). Oncogenes of the second group, on the other hand, are mostly membrane bound or cytoplasmic proteins which are able to provide the rest of the transformation properties to already established cells (10, 11, 13, 14, 26). This is evidenced by the ability of the cells to assume a transformed morphology, grow in an anchorage-independent fashion, and grow as a tumor when injected into a syngeneic animal (11).

Oncogenes of the first category, although unable by themselves to transform established lines, are able to modify their phenotype. Expression of the *myc* oncogene in mouse C3H 10T1/2 cells, for instance, has been shown to result in increased sensitivity to transforming growth factor β (TGF β) (12, 25), as shown by the acquired ability of these cells to form colonies in soft agar in response to TGF β after *c-myc* expression (12). Similarly, Fisher rat 3T3 cells expressing an activated *c-myc* oncogene were rendered anchorage independent in response to epidermal growth factor (EGF) (25). On the other hand, as mentioned above, cells expressing transforming oncogenes, like *v-ras*, grow in agar spontaneously; that is, they both produce transforming growth factors and are able to respond to them (12, 25).

Polyomavirus-transformed cells express three proteins, termed small t (st), middle T (mT), and large T (LT) tumor antigens (6). LT is able to establish primary cells in culture, while mT is able to transform certain already immortalized cell lines, such as rat F111 fibroblasts, to agar growth and tumorigenicity in syngeneic animals (17). However, marked differences have been noted in the ability of different continuous lines to grow in agar in response to mT expression alone (16). A clone of NIH 3T3 cells (termed NmT-1) expressing mT from the dexamethasone-regulatable promoter of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) is totally unable to grow in agar after mT antigen induction, although it can be transformed through expression of IT and st in addition to mT antigen (16). In this communication we present evidence that mT expression in NmT-1 cells increases the responsiveness of these cells to the transforming growth factors produced by

the same parent line rendered neoplastic through the action of the whole transforming region of polyomavirus.

Growth factor secretion by polyomavirus-transformed NIH 3T3 cells. Growth in agar of many transformed cells, including cells transformed by polyomavirus (8), correlates with the ability of these cells to produce and secrete peptides which can confer a transient anchorage-independent growth phenotype to responsive normal cells (7). This was verified in the present system. As shown in Fig. 1A and B, concentrated growth medium conditioned by polyomavirus-transformed NIH 3T3 (py3T3) cells was able to promote the growth of the parent normal line (NIH 3T3) on agar. Approximately 18% of the cells formed colonies larger than 50 μ m after 10 days.

A transforming growth factor that has been reported to be produced by various cultured cells is TGF β (21). Overnight incubation of the conditioned medium with increasing amounts of a neutralizing antibody to TGF β (R&D Systems) drastically reduced the agar growth of the NIH 3T3 cells, indicating that TGF β is present in the conditioned medium and is essential for this phenotypic effect (Fig. 2). Addition of the antibody to the agar suspension at the same time as the growth factors or addition of a twofold excess of growth factors or 5 ng of TGF β per ml resulted in the inability of the antibody to abolish growth stimulation by the conditioned medium at a concentration of 300 μ g/ml, indicating that this inhibition was specific to TGF β and could not be due to nonspecific inhibitors present in the antibody preparation. NIH 3T3 cells did not grow in agar supplemented with TGF β (1 to 5 ng/ml) alone, but a combination of TGF β (5 ng/ml) and EGF (5 ng/ml) did support their anchorage-independent growth. Together, the above results indicate that these two growth factor activities might be present in the conditioned medium and acting synergistically.

The rat fibroblast line NRK-49F is able to grow in soft agar medium only in the presence of TGF β and EGF (19). This property was used to develop a very sensitive assay for the quantitation of these growth factor activities in conditioned medium or tissue extracts (18, 19). In the present study, no growth of NRK-49F cells (American Type Culture Collection) was noted after addition of EGF or TGF β alone or basic or acidic fibroblast growth factor (FGF; 0.2 to 50 ng/ml; Boehringer Mannheim). Therefore, conditioned me-

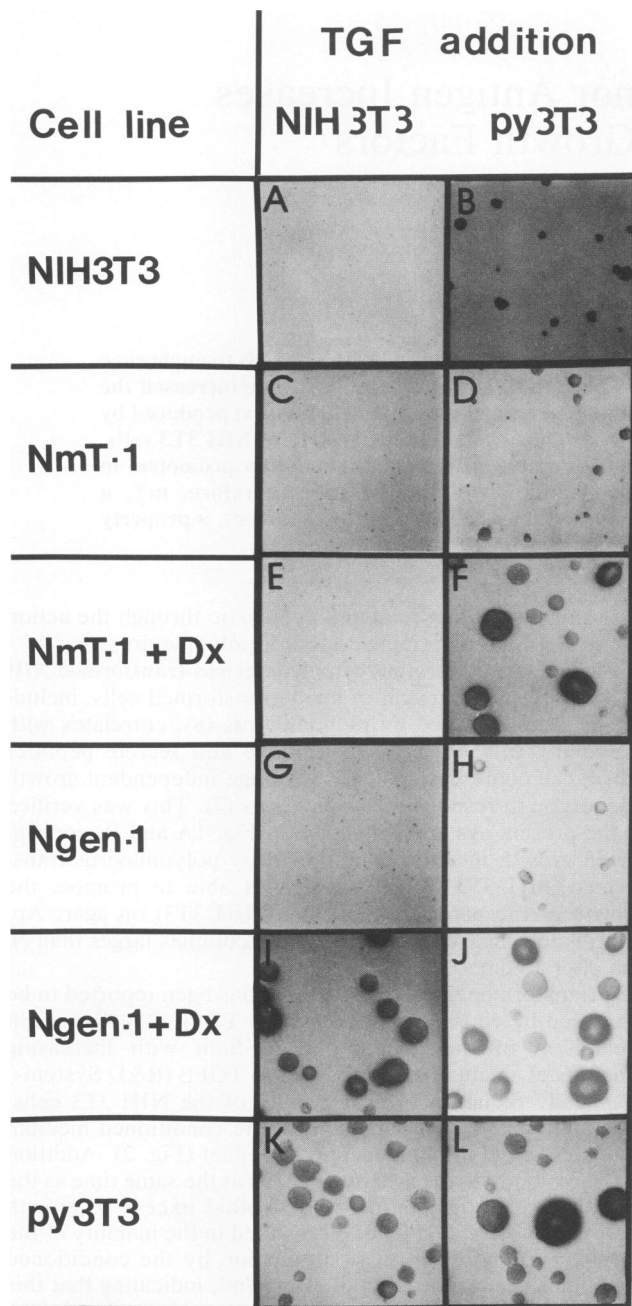


FIG. 1. Growth in agar of NIH 3T3 cells and derivatives upon addition of TGF. For growth factor preparation, subconfluent py3T3 or NIH 3T3 cells were washed twice with serum-free Dulbecco's modified Eagle's medium (DMEM) and grown in the absence of serum for 48 h. The conditioned medium was then collected, clarified by centrifugation, and concentrated 100-fold with an Amicon Ultrafiltrator (5,000 molecular weight cutoff). Agar was subsequently added from a 1.8% suspension to achieve a final concentration of 0.35%, and the DMEM concentration was adjusted with a five-times-concentrated solution. The residual amount of serum present in the conditioned medium concentrates (less than 0.2%) was measured by gel electrophoresis and Coomassie blue staining, and the serum concentration was adjusted to 5% for all plates. NIH 3T3 (A and B), NmT-1 (C to F), Ngen-1 (G to J), and py3T3 (K and L) cells were seeded in 6-cm plates in this soft agar containing concentrated medium conditioned by NIH 3T3 (A, C, E, G, I, and K) or py3T3 (B, D, F, H, J, and L) cells as indicated, on top of a

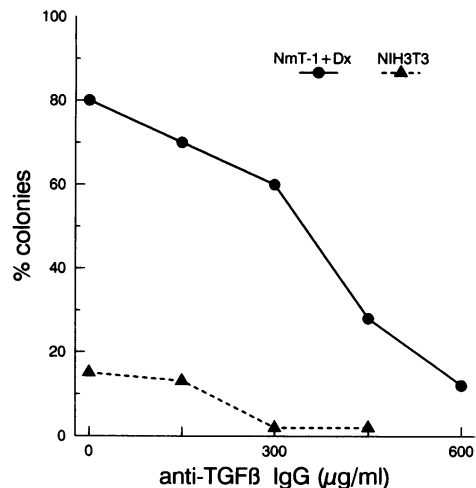


FIG. 2. Inhibition of anchorage-independent growth of NIH 3T3 and induced NmT-1 cells in medium conditioned by py3T3 cells by the addition of a neutralizing antibody to TGFβ. NIH 3T3 and induced NmT-1 cells were plated in agar-containing conditioned medium treated with the indicated amounts of specific anti-TGFβ antibodies. The percentage of cells forming colonies larger than 50 μm was recorded 10 days later. Dx, Dexamethasone.

dium from py3T3 cells was titrated for EGF-like activity (stimulation of anchorage-independent growth of NRK-49F cells in the presence of TGFβ [2 ng/ml] and 10% calf serum) and TGFβ activity (stimulation of anchorage-independent growth of NRK-49F cells in the presence of EGF [5 ng/ml] and 10% calf serum) by comparison of dilution curves of conditioned medium with those of standards of porcine platelet TGFβ or EGF (ICN Biomedicals).

py3T3 cells secreted an average of approximately 50 ng of TGFβ per 10⁸ cells per 48 h and EGF-like activity equivalent to an average of 60 ng of EGF per 10⁸ cells per 48 h (Fig. 3). The corresponding numbers for the NIH 3T3 line were 10 and 12 ng, respectively, in agreement with previous observations that such peptides are also secreted by normal cells (24). Similar quantities were secreted by the NmT-1 line before and after promoter induction with 1 μM dexamethasone for 24 h (17), indicating that mT expression had no effect on growth factor production. This could be at least part of the reason why NmT-1 cells are unable to grow in agar even after full promoter induction (16).

Unlike F2408 cells (8), NIH 3T3 cells were unable to grow in agar after addition of EGF (0.2 to 20 ng/ml) or basic or acidic FGF, alone or in combination with insulin, transferrin, and selenium, indicating that these growth factor combinations, if present in the py3T3-conditioned medium, cannot be solely responsible for the proliferation of the NIH 3T3 cells in soft agar. Consistent with this interpretation, the addition of protamine sulfate (10 to 200 μg/ml) to the agar medium, a procedure which specifically blocks platelet-derived growth factor (PDGF) and FGF binding to their respective receptors (3-5), had no effect on the anchorage-independent growth of py3T3 cells.

layer of 0.6% agar containing the corresponding conditioned medium. A 2-ml amount of the same 0.35% agar medium was added to each plate after 5 days. Pictures were taken 10 days later under low magnification. Dx, Dexamethasone.

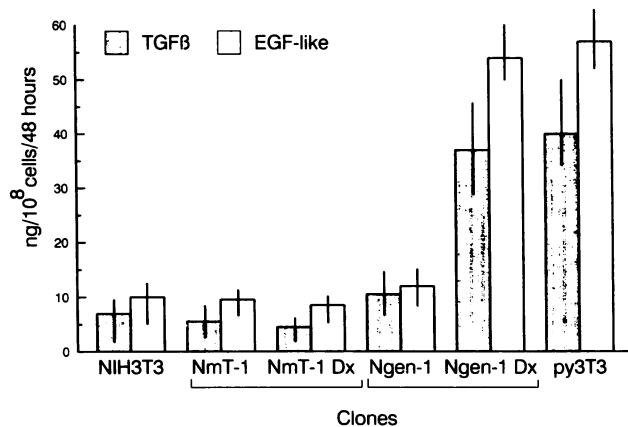


FIG. 3. Growth factor secretion by polyomavirus tumor antigen-expressing NIH 3T3 cells. The quantity of EGF-like activity (stimulation of growth of NRK-49F cells on agar in the presence of TGFβ [2 ng/ml]) and TGFβ activity (stimulation of the growth of NRK-49F cells on agar in the presence of EGF [5 ng/ml]) was calculated by comparing dilution curves of conditioned medium with those of standards of porcine EGF or TGFβ. Each quantitation was conducted four times. Error bars indicate the variation. Dx, Dexamethasone.

mT expression increases the anchorage-independent growth of NIH 3T3 cells in response to growth factors. Previous results indicated a slight increase in the mT-associated tyrosine protein kinase activity in vitro after addition of EGF or 20% serum to mT-expressing Rat-1 cells grown in 0.5% serum (22). Therefore, the possibility that the transforming growth factors secreted by the py3T3 cells affected mT activity in NmT-1 cells was investigated. As shown in Fig. 4, there was no detectable increase in the tyrosine-protein kinase activity of the mT-pp60^{c-src} complex after growth factor treatment of NmT-1 cells growing in 5% serum (Fig. 4, lanes 2 and 3). Similarly, growth factor addition to an NIH 3T3 line expressing the IT, mT, and st antigens under control of the same MMTV LTR promoter (16) (Ngen-1, Fig. 4) or to the py3T3 cells (not shown) revealed no effect on in vitro mT phosphorylation. Moreover, a direct comparison of the in vitro mT-pp60^{c-src} complex kinase activity levels between

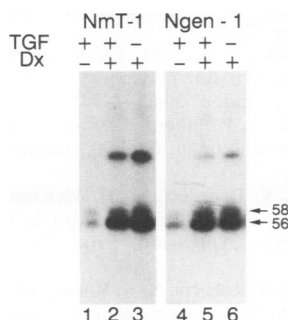


FIG. 4. Comparison of the in vitro phosphorylation of mT from NmT-1 (lanes 1 to 3) and Ngen-1 (lanes 4 to 6) cells in the presence (lanes 2, 3, 5, and 6) and absence (lanes 1 and 4) of dexamethasone (Dx), before (lanes 3 and 6) and after (lanes 1, 2, 4, and 5) the addition of TGFs from medium conditioned by py3T3 cells. Detergent cell extracts were immunoprecipitated with antipolyomavirus ascites fluid (17), and [γ -³²P]ATP was added to washed immunoprecipitates prior to electrophoresis through an 8% polyacrylamide gel. Arrows point to the positions of the 56- and 58-kDa phosphorylated mT forms.

NmT-1 and Ngen-1 (16) (Fig. 4) or a rat F111 line expressing the mT antigen under control of the same MMTV LTR promoter (mT-1 [17]) before and after growth factor addition revealed no significant differences, indicating that the inability of the NmT-1 cells to grow in soft agar medium cannot be explained by a deficiency in mT-associated tyrosine-protein kinase activity. Similarly, examination of the mT-pp60^{c-src}-associated phosphatidylinositol-3 kinase activity levels (16) revealed no detectable increase after growth factor addition for the NmT-1, Ngen-1, or mT-1 line (not shown).

Since the responsiveness of cultured cells to growth factors has been shown to be modulated by viral oncogenes (12, 20, 25), the growth of the NmT-1 line in agar after addition of py3T3 growth factors was examined. As shown in Fig. 1D and F, mT induction in NmT-1 cells resulted in a dramatic increase in their anchorage-independent growth in the presence of the growth factors made by the py3T3 line, approaching the growth of the py3T3 cells themselves. A similar response was elicited with a combination of TGFβ (5 ng/ml) and EGF (5 ng/ml) (not shown), while addition of concentrated medium conditioned by NIH 3T3 cells had no significant effect (Fig. 1A, C, E, G, I, and K). Prior incubation of the py3T3-conditioned medium with the neutralizing antibody to TGFβ abolished this effect, indicating that TGFβ must be at least partly responsible (Fig. 2). The growth of the corresponding line expressing IT, mT, and st from the same MMTV LTR promoter (Ngen-1) as well as the py3T3 clone was also slightly increased after addition of the py3T3-conditioned medium, indicating that although these lines do produce sufficient amounts of and respond to their own growth factors, exogenous addition could further enhance their growth (Fig. 1G to L). Addition of dexamethasone to NIH 3T3 or py3T3 cells had no effect on their soft agar growth either in the presence or in the absence of growth factors (not shown).

To further strengthen the conclusion that mT induction in the NmT-1 cells changes the response to the growth factors rather than the response to the dexamethasone itself, the following experiment was performed. The mT gene was expressed in the NIH 3T3 cells under control of the resident polyomavirus promoter (17) and hygromycin resistance co-selection (16). Twelve such clones were picked and expanded into lines. As expected, these cells did not grow in agar except after growth factor addition. Moreover, addition of dexamethasone to these cells had no effect on their growth in agar, either in the presence or in the absence of growth factors (not shown).

The inability of the mT antigen, a viral tumor gene which is able to transform cells like rat F111 fibroblasts (17), to convert a clone of NIH 3T3 cells to anchorage-independent growth (16) indicated a clear difference in the reaction of different lines to the same oncogene. Other NIH 3T3 clones were variously shown to be transformable by mT alone, to the same (1, 2) or a lesser (15) extent than by the whole early region of polyomavirus. The reaction of this clone of NIH 3T3 cells was especially surprising, since NIH 3T3 cells are considered by many investigators to be easy to transform by a variety of agents (9, 23). Nevertheless, the inability of mT to render this clone anchorage independent made it possible to demonstrate that mT can tremendously increase the responsiveness of these cells to growth factors made by the py3T3 line, as evidenced by the enhanced growth of NmT-1 cells in agar supplemented with these growth factors after mT antigen induction. NIH 3T3 cells expressing the whole transforming region of polyomavirus, on the other hand, secreted higher levels of growth factors and were able to respond adequately to them so that they grew in agar without

any exogenous addition. Therefore, growth factor production and response appear to be two separable properties required for the agar growth of NIH 3T3 cells. mT expression alone increases the responsiveness to growth factors, while IT and st, in combination with mT, increase transforming growth factor production by approximately fivefold, with spontaneous growth as a result. This increase in autocrine factor secretion has a very marked effect on the cellular phenotype, as if a critical threshold of growth factor dose is being exceeded. Endogenous growth factors such as the ones produced by the py3T3 and induced Ngen-1 cells might be more effective than exogenously added ones, since they might stimulate the receptors of the same cell before diffusing into the medium.

As shown in Fig. 1B and D, the low background levels of mT expression in the NmT-1 line (approximately 3%, Fig. 4) were not sufficient to promote agar growth to any measurable extent above the growth of the parent NIH 3T3 line after TGF addition. This is consistent with the evidence from a different system, rat F111 cells, in which a high mT expression level is necessary for agar growth as opposed to other transformation-related properties, like the formation of foci overgrowing a monolayer of normal cells or the manifestation of transformed morphology on plastic (17).

In contrast to the effect of mT on NIH 3T3 cells, *ras* expression in C3H10T1/2 cells did not cause an increased ability for agar growth after growth factor addition even when these cells were unable to grow under anchorage-independent conditions (24). Moreover, contrary to *ras*-expressing cells (12, 25), mT did not appreciably increase the amount of TGFs produced, and hence the NmT-1 cells were unable to grow in agar spontaneously.

The similarity of the response of mT-expressing cells to transforming growth factors with cells overexpressing the *myc* gene might be related to the fact that mT expression in NmT-1 cells triggers the transcription of the *c-myc* and *c-fos* oncogenes (27). In any case, this is the first instance in which a transforming oncogene, the polyomavirus mT antigen, is shown to enhance the sensitivity of an established cell line to growth factors.

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