Polyomavirus Transforms Rat F111 and Mouse NIH 3T3 Cells by Different Mechanisms

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Polyomavirus middle tumor antigen (mT) was expressed in a line of mouse NIH 3T3 cells under control of the dexamethasone-regulatable mouse mammary tumor virus promotor. Contrary to rat F111 cells which were rendered anchorage independent by mT expression alone (L. Raptis, H. Lamfrom, and T. L. Benjamin, Mol. Cell. Biol. 5:2476–2487, 1985), mT-producing NIH 3T3 cells were unable to grow in agar even after full mT induction. The mT:pp60^{c-src}-associated phosphatidylinositol kinase was activated in these cells to a degree similar to that in fully transformed cells expressing the small and large T antigens, in addition to mT. We therefore propose that the stimulation of this phosphatidylinositol kinase, although apparently necessary, is not sufficient for transformation of NIH 3T3 cells by polyomavirus.

Among the three tumor antigens encoded by polyomavirus, middle T (mT) is thought to be the principal protein involved in transformation (34). In the cell, mT associates with the carboxy end of pp60^{c-src}, the cellular homolog of the transforming protein of Rous sarcoma virus, pp60^{v-src} (8, 14). This interaction results in an increase in the tyrosine protein kinase activity of pp60^{c-src} (6), partly because mT stabilizes a form of this enzyme which is not phosphorylated at position 527, the major tyrosine phosphorylation site of pp60^{c-src}, which is thought to negatively regulate kinase activity of the enzyme (10, 11, 20). It has been proposed that an in vivo substrate of this activated tyrosine kinase is an 85-kilodalton (kDa) phosphoprotein with L-α-phosphatidyl-inositol (PI) kinase activity (13, 21). Extensive genetic and biochemical evidence has implicated this association of mT with pp60^{c-src} in the mechanism of transformation by polyomavirus (7, 8, 12, 13, 22, 33). It has also been shown that p62^{c-yes} (24) and fyn (S. Cheng, personal communication; J. B. Bolen and K. C. Robins, unpublished observations) cellular oncogenes bind to mT, although the relationship of this association with transformation has not been established.

Although mT is necessary for transformation, it is not always sufficient by itself. Transformation of nonestablished cells by means of cloned mT cDNA expression requires complementation by large T (25, 31), while the contribution of the small or large T to the transformed phenotype of established rat fibroblasts has been a matter of controversy (18, 28). One set of results indicates that although the Fisher rat cell line FR3T3 can be transformed to anchorage independence by expressing the mT gene alone, the acquisition of serum independence requires the additional presence of at least the N-terminal portion of large T (31, 32), while according to other reports, serum or anchorage independence in cells already expressing small T and mT is not necessarily correlated with the levels of large T or fragments thereof (18, 28).

The role of mT in the transformation of mouse NIH 3T3 cells is equally unclear; some reports, by expressing this gene under control of the resident polyomavirus promoter, show that mT is sufficient for transformation of these cells

(9, 15), while other data indicate that small T is necessary, in addition to mT, for the transformation of NIH 3T3 cells to anchorage independence (27).

The ability to regulate the expression of polyomavirus tumor antigens in Fisher rat F111 cells (16), by placing them under control of the dexamethasone-inducible promoter of the mouse mammary tumor virus (MMTV) long terminal repeat, facilitated the resolution of the above discrepancies; mT antigen alone, if it is stably expressed to adequate levels, is in fact able to transform these cells to the anchorage-independence phenotype (30). Besides, the ability to control the level of expression, through control of the inducer concentration in the medium, permitted for the first time the correlation between the quantity of mT present in the cell and a given phenotype to be established; different phenotypic responses required different levels of mT antigen induction, with morphological transformation, focus formation, and anchorage-independent growth requiring increasing amounts of mT antigen. Therefore, differences observed before could be explained on the basis of clonal variations in the original cell population or differences in the level of mT antigen expression in the transfected cell lines.

Given the advantages of regulated gene expression, we decided to study the properties of NIH 3T3 cells expressing mT under the control of the dexamethasone-inducible MMTV long-terminal-repeat promoter, as a step toward understanding the role of mT in the transformation of this cell line.

MATERIALS AND METHODS

Materials. Geneticin (G418 sulphate) was from GIBCO Laboratories, Grand Island, N.Y., and hygromycin B was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. PI and L-α-phosphatidylinositol-4-monophosphate were purchased from Sigma Chemical Co., St. Louis, Mo. (catalog no. P2517 and P9638, respectively). [^{35}S]Methionine trans-label grade) and [γ-^{32}P]ATP (2,000 Ci/mmol) were purchased from ICN Pharmaceuticals Inc., Irvine, Calif. Electrophoresis reagents were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and silica gel thin-layer chromatography plates were obtained from EM Science, Federal Republic of Germany (catalog no. 5553).

Cell lines, culture techniques, and tumor antigen analysis.

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Cell culture and T-antigen analysis were performed essentially as described previously (30). In all in vitro phosphorylation experiments, the gels had been treated with alkali prior to autoradiography. Induction of the tumor antigens for biochemical analyses was achieved by overnight incubation in the presence of 10^{-6} M dexamethasone (30).

Production of mouse NIH 3T3 cell lines inducible for mT or the corresponding genomic piece of polyomavirus DNA. pBR322-based plasmids were constructed, containing the MMTV long-terminal-repeat promoter upstream from the coding region of mT (derived from cDNA) or the corresponding genomic piece of DNA (30). The plasmids were separately introduced with G418 resistance selection in the NIH 3T3 cells, exactly as described for the rat F111 cells (30). A 200-μg/ml concentration of G418 (rather than 400 μg/ml, which was used for the F111 lines) was found to be sufficient for the selection of resistant NIH 3T3 clones.

PI kinase assays. PI kinase assays were performed essentially as described by Whitman et al. (36) and Kaplan et al. (21, 22), with minor modifications. In brief, clarified 1% Nonidet P-40 extracts from approximately 5×10^6 cells were precipitated with anti-polyomavirus ascites fluid. Immunoprecipitates were washed three times with 0.5% Nonidet P-40 to remove all traces of type II PI kinase (36) and twice with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.2). Immune complexes were suspended in 40 μl of 20 mM HEPES (pH 7.2)–0.2-mg/ml PI (dispersed by sonication) and were incubated on ice for 10 min. The reaction was initiated by the addition of 5 mM $MnCl_2$ and 10 μCi of $[\gamma^{-32}P]ATP$. After incubation for 15 min at 20°C, the reaction was stopped with the addition of 100 μl of 1 M HCl; phospholipids were extracted with 200 μl of chloroform-methanol (1:1) and were resolved by chromatography on silica gel plates in a chloroform–methanol–4 M NH_4OH (9:7:2) mixture. Cold L-α-phosphatidylinositol-4-monophosphate served as a marker.

Expression of the 808A polyomavirus mutant DNA in the G418-resistant NmT-1 cells. The pY3 plasmid, containing the hygromycin B phosphotransferase gene (3), was purchased from the American Type Culture Collection, Rockville, Md. The 808A polyomavirus mutant DNA, cloned in the BamHI site of the pUC19 plasmid, was a gift of R. Freund and D. Talmage (Harvard Medical School). Transfections were conducted as described previously (30), by using 10 μg of total DNA per 2×10^6 cells, at a polyomavirus 808A/pY3 ratio of 50:1. About 12 h after exposure to the DNA, the cells were trypsinized and replated at a 1:10 dilution. On day 3 after transfection, the medium was replaced with fresh medium containing 10% calf serum and 100 μg of hygromycin B per ml. This medium was changed every 3 to 4 days, and clones were picked about 10 days later. These were maintained in medium containing 100 μg of hygromycin B per ml for approximately 60 days, after which the drug was completely removed. Clones obtained this way remained phenotypically unchanged for more than a year in culture.

Precipitation of cell extracts with the 1G2 monoclonal antibody to phosphotyrosine. The Sepharose-coupled 1G2 monoclonal antibody was a gift of J. Bell (McGill University) and was used as described elsewhere (19, 29). Briefly, extracts from 5×10^6 cells (prepared in a solution of 1% Nonidet P-40–10 mM Tris–5 mM EDTA–50 mM NaCl–30 mM sodium pyrophosphate–50 mM NaF–100 μM sodium orthovanadate–0.1% bovine serum albumin [pH 7.6]) were precipitated with 1G2-coated Sepharose beads by incubation on ice for 3 to 4 h. Nonadsorbed material was eliminated by washing five times with the same extraction buffer and twice

with kinase buffer (20 mM Tris hydrochloride [pH 7.5]–5 mM $MnCl_2$). The reaction was initiated by the addition of 100 μCi of $[\gamma^{-32}P]ATP$ in 100 μl of kinase buffer, followed by incubation at room temperature for 30 min. Finally, the phosphotyrosine-containing proteins were specifically eluted with 100 mM phenylphosphate and were run on a sodium dodecyl sulfate-polyacrylamide gel as described elsewhere (30).

Transformation assays. (i) Agar growth. Approximately 10^4 cells were suspended in 2 ml of 0.36% Bacto-Agar (Difco Laboratories, Detroit, Mich.)-containing Dulbecco modified Eagle medium supplemented with 3% calf serum, on top of a feeder layer of the same medium containing 0.7% agar, in 6-cm petri dishes. To avoid potential toxicity problems, induction was performed through the addition of 0.5×10^{-6} M dexamethasone (rather than 10^{-6} M) to both layers. Growth was recorded 7 days later.

(ii) Focus formation assays. Approximately 200 inducible cells were plated along with 2×10^4 normal NIH 3T3 cells into 6-cm dishes in medium containing 5% calf serum and 0.5×10^{-6} M dexamethasone, where indicated. At 7 to 8 days later, the cells were fixed, stained with Coomassie blue, and photographed.

RESULTS

Production of NIH 3T3 polyomavirus tumor antigen-inducible cell lines. Mouse NIH 3T3 cells were transfected with a cloned DNA consisting of the polyomavirus mT gene (or the corresponding genomic piece of DNA) located downstream from the MMTV long-terminal-repeat promoter (plasmids pSVMmT and pSVMgen, respectively) (30), along with a plasmid conferring resistance to G418, serving as a selectable marker (pMoNeo) (30). Individual colonies were picked without regard to morphology. These were then propagated in the presence or absence of 0.5×10^{-6} M dexamethasone and were screened for levels of mT-associated kinase activity in a standard immunoprecipitation assay, as described previously (30). As in the case of the transfected F111 clones (30), the uninduced and induced levels of mT varied from clone to clone, while several clones displayed fully induced levels comparable to that of the NIH 3T3 derivative cell line which has been transformed by whole polyomavirus (py6) (2). Of 228 pSVMmT-transfected lines, only 5 showed inducible levels of mT-associated kinase activity higher than 20% of the level of typical polyomavirus-transformed NIH 3T3 cells, compared with 4 of 136 pSVMgen transfectants. One representative each of the mT- or genomic DNA-expressing clones NmT-1 and Ngen-1, respectively, was chosen for further study.

NmT-1 cells formed foci but were unable to grow in agar. The ability of either the NmT-1 or Ngen-1 cell line to form dense foci on monolayers of normal NIH 3T3 cells was clearly dependent on mT induction. Both lines formed foci with equal efficiency, plateauing at an induction level of approximately 20% of the maximum (30) (Fig. 1A through D). However, the NmT-1 line was unable to grow in agar even after full mT induction (Fig. 1F). This is in agreement with the results of Noda et al. (27), who found that NIH 3T3 cells constitutively expressing the mT gene did not grow in agar to the same extent as cells simultaneously expressing mT and small T. This was clearly not the case with the rat F111 cells, since the mT gene alone expressed from the same MMTV promoter was able to transform these cells to anchorage independence (30), although higher expression levels were necessary for agar growth than for foci formation
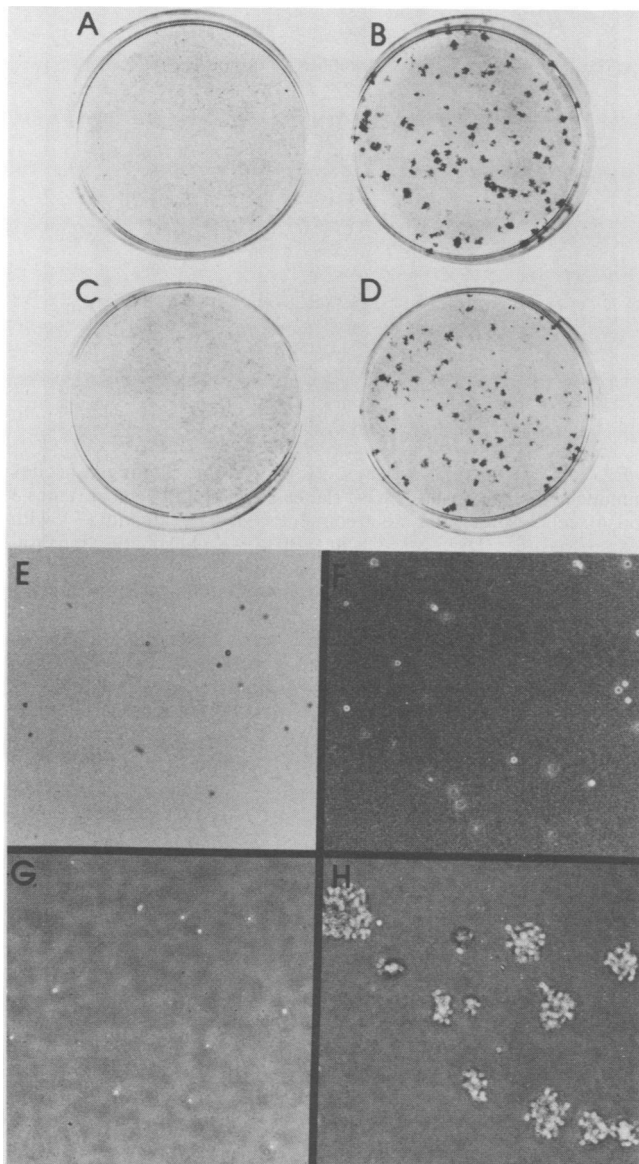
FIG. 1. Transformation assays. Foci formation of NmT-1 (A and B) and Ngen-1 (C and D) cells without (A and C) or with (B and D) dexamethasone addition and agar growth assays of NmT-1 (E and F) and Ngen-1 (G and H) cells without (E and G) or with (F and H) dexamethasone addition are shown.
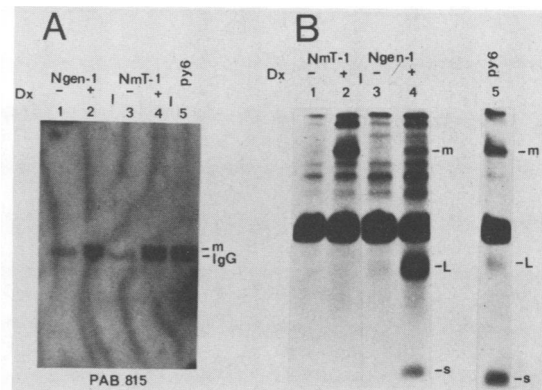

FIG. 2. Quantitation of mT protein. (A) PAb 815 antibody immunoblot analysis (4, 5) of NmT-1 (lanes 3 and 4), Ngen-1 (lanes 1 and 2), and py6 (lane 5) cells treated (+) or not treated (−) with dexamethasone (Dx). The positions of mT antigen (m) and rat immunoglobulin heavy chain (IgG) are indicated. (B) Tumor antigen analysis of the same cells labeled in vivo with [^{35}S]methionine in the presence or absence of the inducer. NmT-1 cells without (lane 1) or with (lane 2) dexamethasone, Ngen-1 cells without (lane 3) or with (lane 4) dexamethasone, and py6 cells (lane 5) are shown. The positions of mT (m), large T fragment (L), and small T (s) are indicated.

(30). Therefore, the reasons for this difference were investigated further.

First, to exclude the possibility that the transfecting mT DNA was somehow mutated, the same DNA preparation was transfected into F111 cells and was found able to transform them to anchorage independence, as described previously (30). Second, several other NIH 3T3 lines expressing the mT gene (or the corresponding genomic piece of DNA) under MMTV promoter control were produced and tested for agar growth. Just like the NmT-1 line, at least three independently produced mT-expressing lines did not grow in agar, while two genomic lines grew in agar only after induction (data not shown). This evidence makes it highly unlikely that the inability of the mT-expressing lines to be anchorage independent is due to random clonal variation in

the initial cell population. Finally, in a separate control experiment, the large and small T antigens were simultaneously expressed in the NmT-1 line through transfection with 808A polyomavirus mutant DNA (26), in which the unique mT splice site had been destroyed, and cotransfection with the plasmid pY3, conferring resistance to hygromycin B (3). The resistant clones were screened for the presence of small and large T antigens by [^{35}S]methionine labeling and immunoprecipitation and were tested for agar growth. At least two such clones constitutively expressed small and large T antigens and grew in agar only after mT induction. This would indicate that the mT gene present in the NmT-1 cells was not mutated or otherwise rendered inactive during the transfection and selection of the NmT-1 line.

The NmT-1 cell line contains higher levels of mT protein than the Ngen-1 cell line. To compare the steady-state levels of mT antigen expression after full promoter induction in the NmT-1 and Ngen-1 lines, the abundance of mT antigen was determined by quantitative immunoblot analysis with the PAb 815 monoclonal antibody to mT (4, 5). The amount of mT protein present was at least twofold higher in the NmT-1 line than in the Ngen-1 line (Fig. 2A). Similarly, estimation of the tumor antigen expression level by metabolic labeling with [^{35}S]methionine followed by immunoprecipitation with anti-T antigen ascites fluid and gel electrophoresis (30, 33) showed that the NmT-1 line contains approximately two times more [^{35}S]methionine-labeled mT than the Ngen-1 line, while for the latter, small T as well as a large T fragment were clearly detectable after promoter induction (Fig. 2B).

The in vitro kinase activity of the mT:pp60^{c-src} complex was the same in NmT-1 and Ngen-1 cells. The transformation parameters and tumorigenicity of the mT-expressing F111 cells correlated with the level of mT phosphorylated by pp60^{c-src} in immune complexes and not with the total amount of mT as determined by metabolic labeling (30). Therefore, to exclude the possibility that the Ngen-1 line had higher levels of "active" mT than did the NmT-1 line, the levels of in vitro phosphorylation of mT after precipitation with
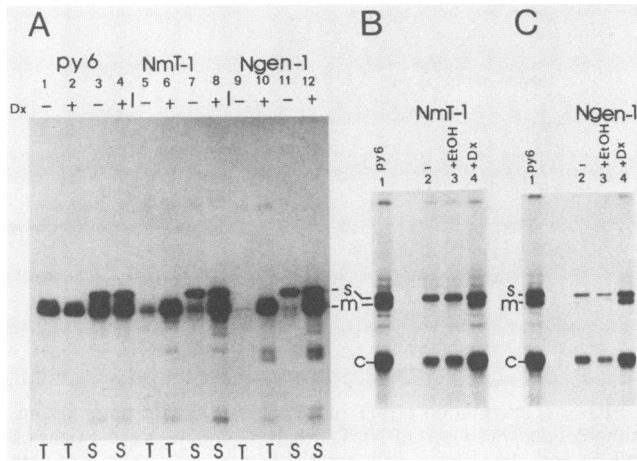
FIG. 3. Analysis of mT antigen-associated protein tyrosine kinase activity. (A) RIPA buffer-washed immunoprecipitates (6) prepared from extracts of the py6 (lanes 1 to 4), NmT-1 (lanes 5 to 8), and Ngen-1 (lanes 9 to 12) cell lines against anti-T antigen ascites fluid (lanes 1, 2, 5, 6, 9, and 10) or monoclonal antibody 327 to pp60^{src} were incubated with [γ-^{32}P]ATP and magnesium before gel electrophoresis. Symbols: +, cells treated with dexamethasone (Dx); −, untreated. T, Anti-polyomavirus tumor antigen ascites fluid; S, monoclonal antibody 327 to pp60^{src}. (B and C) Phosphorylation of caseine by monoclonal antibody 327 immune complexes from NmT-1 (B, lanes 2 to 4), Ngen-1 (c, lanes 2 to 4), or py6 (B and C, lanes 1) cell RIPA buffer lysates after no treatment (B and C, lanes 1 and 2), treatment with ethanol (B and C, lanes 3), or treatment with dexamethasone (B and C, lane 4). The positions of the 56-kDa mT (m), pp60^{c-src} (s), and alpha-casein (c) are indicated.
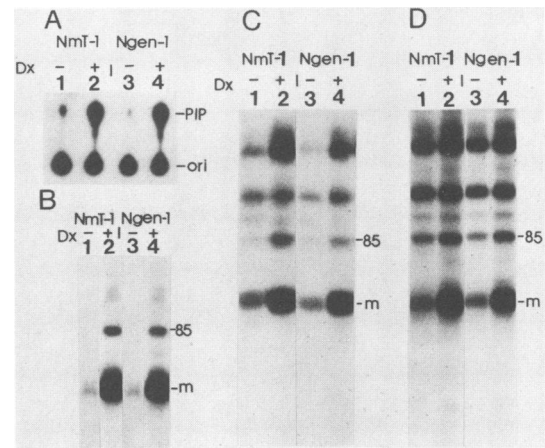

FIG. 4. (A) PI kinase assays (21, 36) on anti-T antigen ascites immune complexes from NmT-1 (lanes 1 and 2) and Ngen-1 (lanes 3 and 4) cell lysates after no treatment (−) or treatment (+) with dexamethasone (Dx). ori, Origin; PIP, L-α-phosphatidylinositol-3-monophosphate. (B) In vitro phosphorylation of mT (m) and the 85-kDa PI kinase (85). Anti-mT immunoprecipitates from NmT-1 (lanes 1 and 2) and Ngen-1 (lanes 3 and 4) cell lysates, without (−) or with (+) dexamethasone treatment were washed and incubated with [γ-^{32}P]ATP and magnesium before gel electrophoresis. (C) In vitro phosphorylation of phosphotyrosine-containing proteins precipitated with the 1G2 monoclonal antibody (19). Lanes are the same as described above. Cells were grown in low (1% calf) serum. (D) Same as for panel C, except cells were grown in high (10% calf) serum before testing.

anti-polyomavirus ascites fluid before and after full induction with 10^{-6} M dexamethasone in the NmT-1 or Ngen-1 cell line were examined. Both lines contained very similar levels of pp60^{c-src}-associated mT (Fig. 3A). Uninduced levels were less than 5%, while fully induced levels were roughly 100% that of the py6 line (Fig. 3A, lanes 1, 2, 5, 6, 9, and 10). Similarly, the levels of total pp60^{c-src} autophosphorylation in immunoprecipitates against the anti-pp60^{src} monoclonal antibody 327 (6) were the same in all cell lines, regardless of the presence or absence of mT antigen expression (Fig. 3A, lanes 3, 4, 7, 8, 11, and 12). Moreover, the phosphorylation of caseine by antibody 327 immunoprecipitates before or after polyomavirus tumor antigen induction was the same for both inducible lines (Fig. 3B and C). These results indicate that the presence of the large and small T antigens does not influence the ability of pp60^{c-src} to phosphorylate mT, itself, or exogenous substrates and that the phenotypic differences observed could not be attributed to inherent differences in pp60^{c-src} kinase activity levels in these two lines.

Since the NmT-1 cells expressed an approximately two-fold higher level of mT protein than the Ngen-1 cells (Fig. 2A and B) and since the in vitro mT phosphorylation levels were the same for both lines, it follows that a fraction of the mT molecules immunoprecipitated from the NmT-1 cells was not phosphorylated by pp60^{c-src}. Consistent with these results, a similar overexpression of mT which is not phosphorylated by or associated with pp60^{c-src} has been observed with certain rat F111 cell lines following the same transfection and screening protocol (e.g., line gen-1) (30).

As a control, dexamethasone addition to the virally transformed py6 line (2) had no effect on mT or pp60^{c-src} in vitro phosphorylation (Fig. 3A, lanes 1 to 4).

Stimulation of the 85-kDa mT:pp60^{c-src}-associated PI kinase was the same in the NmT-1 and Ngen-1 cells. The capacity of

some polyomavirus mutants which are defective in cell transformation (e.g., dl23) to stimulate the 85-kDa PI kinase associated with the mT:pp60^{c-src} complex is severely impaired, although the same mutants are apparently able to stimulate to some extent the tyrosine protein kinase activity of pp60^{c-src} (13, 21, 22). Therefore, to determine the degree of PI kinase activation in cells which are not fully transformed, although they express wild-type mT, the extent of phosphorylation of PI to phosphatidylinositol-3-monophosphate by polyomavirus anti-T antigen immunoprecipitates was examined next.

Sonicated PI and [γ-^{32}P]ATP were added to washed anti-mT immunoprecipitates (36) prepared from NmT-1 and Ngen-1 cells with or without dexamethasone treatment (see Materials and Methods). The amount of ^{32}P incorporation into phosphatidylinositol-3-monophosphate (35) was the same in both lines (Fig. 4A). Furthermore, the in vitro phosphorylation of the 85-kDa phosphoprotein, which correlates with increased PI kinase activity, on immunoprecipitates prepared against polyomavirus anti-T antigen ascites fluid (Fig. 4B) or the 1G2 monoclonal antibody to phosphotyrosine (19) was very similar for both lines (Fig. 4C and D). Since the 85-kDa PI kinase is also apparently stimulated by the activated platelet-derived growth factor receptor (21), in high-serum (hence high-PDGF) medium the 85-kDa band was present even in the absence of mT (Fig. 4D). In low-serum medium, however, this band appeared only after mT induction in both lines (Fig. 4C). These results indicate that the phenotypic differences observed cannot be explained on the basis of different PI kinase levels in the two lines.

As expected, the PI kinase levels in both the genomic (e.g., line gen-1) (30) and the mT DNA (e.g., line mT-1) (30)-transformed F111 cells were the same, regardless of the

presence of the large or small T antigen (not shown). Besides, the F111-derivative lines contained at full induction slightly lower amounts of in vitro-phosphorylated mT and associated PI kinase activity than did the NIH 3T3 cells, indicating that insufficient PI kinase levels cannot be the reason for the inability of the NmT-1 cells to grow in agar.

DISCUSSION

Although the physiological function of the phosphatidyl-inositol-3-monophosphate produced by the $mT:pp60^{c-src}$-associated PI kinase is unknown (34), it still appears that the $mT:pp60^{c-src}$ complexes are directly involved in the alteration of the polyphosphoinositide metabolism of the cell and therefore might modulate signals from various mitogen receptors, leading to cell transformation. Consistent with this observation, the ability of polyomavirus mutants (e.g., $dl23$) to stimulate this PI kinase correlates somewhat more closely with their transforming potential than the mere association of mT and $pp60^{c-src}$ (13, 21, 22). The present data further indicate that stimulation of the PI kinase activity even by wild-type mT, although apparently necessary, is clearly not sufficient for transformation of NIH 3T3 cells. Some other biochemical change caused by the small or large T antigen is needed, in addition to mT, for agar growth of these cells. This is in contrast to the rat F111 cells, which were found to be totally transformable to anchorage independence and tumorigenicity by mT alone (30).

What the exact role of the small or large T antigen might be, in conjunction with mT, in the transformation of the NIH 3T3 cells is at present unclear. Examination of the ^{125}I-epidermal growth factor binding indicated a relatively small but reproducible increase in binding in the Ngen-1 line after induction, while the increase in the NmT-1 line was insignificant (data not shown). This increase points to the possibility that the small or large T present in the Ngen-1 line might be inducing the expression of a transforming growth factor alpha-like receptor which might in turn be needed for the cells to respond to the secreted transforming growth factors (1, 17, 23) and become anchorage independent. The ^{125}I-epidermal growth factor binding of the F111 cell derivatives was very low, either in the presence or absence of induction, indicating that transformation of the F111 cells by polyomavirus might not involve epidermal growth factor-like receptors. The reasons for this difference are not clear at the moment; nevertheless, the present data indicate that attempts at correlating a biochemical property with the transforming ability of a polyomavirus mutant should take into consideration at least these two types of cell lines as targets.

ACKNOWLEDGMENTS

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