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# Regulation of Cellular Phenotype and Expression of Polyomavirus Middle T Antigen in Rat Fibroblasts

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**Polyoma middle T antigen (mT) was expressed in rat F-111 cells under control of the dexamethasone-regulatable mouse mammary tumor virus promoter. Graded phenotypic responses to levels of mT induction by the hormone were seen, with morphological transformation, focus formation, and anchorage-independent growth requiring increasing levels of mT expression. The ability of different clones to form tumors reflected their maximum level of induction of mT-associated kinase and their ability to grow in soft agar. Expression of transformation parameters and tumorigenicity correlates with the level of mT phosphorylated by pp60$^{c\text{-}src}$ in immune complexes and not with the total amount of mT determined by metabolic labeling. We suggest that cellular factors regulate mT activity by forming a kinase-active fraction of mT molecules that controls the transformed state.**

The ability to regulate the expression of an oncogene should facilitate efforts to understand its mode of action. At least two possibilities exist for experimentally regulating the activity of oncogenes: (i) the use of temperature-sensitive mutants of oncogenic viruses and (ii) the construction of DNAs that express oncogenes under the control of a regulatable promoter.

In the case of polyomavirus, two genes in the early region of the viral DNA play roles in transformation. The *tsa* gene, encoding the large T antigen, is involved in the initiation of transformation. *tsa* mutants may, under certain conditions, act to give temperature-dependent expression of the transformed state (33). Expression of the large T antigen can confer serum independence of growth and lead to the establishment of primary rodent cells (6, 7, 23). The *hr-t* gene, encoding both the small and middle T antigens, is required for the expression of essentially all phenotypic changes that accompany transformation (2). No *ts* mutants in the *hr-t* gene have been isolated, although a cold-sensitive mutation affecting the middle T antigen (mT) was recently described (36). Transfection with cDNAs for individual T antigens has shown that mT alone can transform established rat fibroblasts (37), whereas large T along with mT is required for full transformation of primary cells (22).

As a step toward understanding the effects of mT in transformation, we constructed plasmids in which expression of this protein in transfected cells can be regulated. Polyoma sequences encoding mT were cloned into an expression vector, placing them under the control of the dexamethasone-inducible promoter from the mouse mammary tumor virus long terminal repeat (MMTV-LTR; 18). Similar approaches have recently been reported with v-*ras* (15, 16), v-*mos* (20), and v-*src* (17). Such plasmids encoding mT were transfected into established rat fibroblasts to regulate the synthesis of mT and study its effects on various aspects of cell behavior. The results show quantitative regulation of expression of different parameters of transformation in response to graded levels of mT induction by the hormone. Anchorage-independent growth required the most mT expression, and morphological transformation required the least, with focus formation on monolayers being intermediate. Formation of tumors in syngeneic hosts depends on mT expression roughly to the same degree as anchorage-independent growth. In addition, we have shown that the expression of transformation and tumorigenicity closely follows the level of mT measured as a substrate for pp60$^{c\text{-}src}$ in in vitro kinase assays of immune complexes rather than the overall level of mT protein in the cell. We discuss how these results differ from and extend earlier work designed to relate particular features of transformation to levels of oncogene activity.

## MATERIALS AND METHODS

**Materials.** Restriction enzymes were purchased from New England BioLabs, Inc., [$^{35}$S]methionine (400 to 600 Ci/mmol) was purchased from New England Nuclear Corp., and [$\gamma$-$^{32}$P]ATP (2,000 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc. Electrophoresis reagents were obtained from Bio-Rad Laboratories. G418 was from GIBCO Laboratories (Geneticin, catalog no. 860-1811).

**Culture techniques.** Cells were grown on plastic dishes with Dulbecco modified Eagle medium, containing 5% calf serum and antibiotics. Passage of cultures was done with a trypsin (0.025% [wt/vol])-versene (0.2% [wt/vol]) solution after the cells were washed with phosphate-buffered saline (144 mM NaCl, 2.7 mM KCl, 15 mM sodium phosphate buffer [pH 7.2], 0.92 mM $CaCl_2$, 0.49 mM $MgCl_2$).

**Cells.** NIH-3T3 mouse cells and Fisher rat F-111 cells were described previously (10).

**Plasmid constructions.** The pSVMdhfr plasmid (18) was cut with *Bgl*II (complete) and *Hin*dIII (partial), and a 5.6-kilobase fragment corresponding to the whole plasmid minus

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‡ Hildegard died on 28 August 1984, among her closest friends.

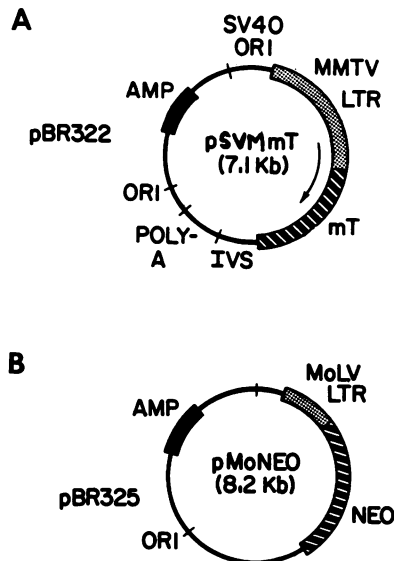


FIG. 1. (A) Plasmid map of the polyoma mT cDNA expression vector utilizing the MMTV-LTR promoter. (B) Plasmid map of the neomycin (G418) resistance gene expression vector utilizing the Moloney leukemia virus-LTR promoter.

the *dhfr* (dihydrofolate reductase) gene was separated on a 1% agarose gel and electroeluted (piece A).

A plasmid DNA containing a polyoma mT cDNA cloned in PBR322 was cleaved with *Hae*II (nucleotide 95) and *Eco*RI (nucleotide 1560). After electroelution, *Hin*dIII linkers were added at the *Hae*II site, and *Bgl*II linkers were added at the *Eco*RI site (piece B).

Pieces A and B were ligated, generating a plasmid which had the mT gene under control of the MMTV promoter in the place of the *dhfr* gene (Fig. 1A).

**Plasmid transfections.** Supercoiled plasmid DNA, without added carrier DNA, was introduced into F-111 cells grown in Linbro trays (10 μg of total DNA per $2 \times 10^6$ cells) by the calcium phosphate precipitation technique of Graham and Van der Eb (14), with a glycerol shock after 4 h (21). The two plasmids were used at a ratio of 100 pSVM-mT:1 pMoneo. Lower ratios resulted in more clones, fewer of which, however, expressed the mT gene. No dexamethasone was added during transfection. About 20 to 24 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 Dulbecco modified Eagle medium containing 10% calf serum and 400 μg of G418 per ml (taking into account that the solid material was only 46% G418). This medium was changed every 4 to 5 days. Clones were picked about 10 days later, trypsinized in cloning cylinders, and transferred to 3-cm petri dishes in medium with 400 μg of G418 per ml. The cells were subsequently maintained in a medium containing 200 μg of G418 per ml for approximately 60 days, after which time the drug was completely removed. Clones grown this way remained phenotypically unchanged for more than a year in culture.

**T antigen analysis.** Procedures for [$^{35}$S]methionine labeling of cells, immunoprecipitation, in vitro kinase reaction, and analysis of T antigens were as described previously (30, 34). T antigens were resolved on NaDodSO$_4$ 10% acrylamide gels. Electrophoresis was done at 60 V for 18 h. Special precautions were taken to assure a quantitative evaluation of T antigen levels, especially that of mT. In preliminary experiments, 5 μl of an anti-T ascites fluid was found sufficient to precipitate the mT from a 6-cm petri dish of polyomavirus-transformed (pyF-111) cells. For quantitation purposes, 20 μl of the same ascites was used per 6-cm petri dish. Also, to assure that the ATP was not limiting in the kinase reaction, cold ATP was added to bring the total concentration of γ-$^{32}$P plus cold ATP to $2 \times 10^{-7}$ M. After electrophoresis, the gels were treated with 1 M NaOH at 60°C for 1 h, dried, and exposed to X-ray film. For quantitation, bands corresponding to the 56,000-molecular-weight (56K) and 58K mT were cut out from the gel and counted in a scintillation counter, or densitometry was performed on multiple exposures of the gel.

## RESULTS

**Construction of MMTV-polyoma recombinant plasmids.** The *Pst*I fragment from the left end of the MMTV proviral DNA encompasses an entire LTR except for 5 to 10 base pairs at the extreme 5′ end (38, 40). The cap site of the MMTV RNA maps to a position 271 nucleotides upstream of the 3′ end of the *Pst*I fragment. The pSVMdhfr plasmid (18) contains the entire *Pst*I fragment of the MMTV-LTR and the mouse *dhfr* gene downstream of this promoter and regulatory region. This vector also contains sequences from the pBR322 plasmid which allow cloning and propagation in *Escherichia coli*, as well as sequences derived from SV40 which contain RNA processing signals allowing production of a functional mRNA for *dhfr* within mammalian cells (35). The *dhfr* gene was excised from this vector by cleavage at the *Bgl*II and *Hin*dIII sites and replaced by a polyomavirus restriction fragment including the polyoma sequences from *Hae*II (nucleotide 95) to *Eco*RI (nucleotide 1560), obtained from a plasmid containing a polyoma mT cDNA (37) (Fig. 1A). This construction was achieved by adding *Hin*dIII linkers to the *Hae*II site and *Bgl*II linkers to the *Eco*RI site. This MMTV-mT gene fusion, designated pSVM-mT, allows transcription to initiate at nucleotide 1182 of the MMTV-LTR and proceed through the mT gene. A plasmid containing the corresponding genomic piece of polyomavirus DNA, designated pSVM-gen, was also constructed. These polyoma plasmids were used in conjunction with a plasmid derived from the transposon Tn*5*, which contains the gene for resistance to the neomycin derivative, G418. For efficient expression in mouse or rat cells, the neo gene in this plasmid, pMoneo, is under control of the Moloney leukemia virus LTR promoter (11; a gift of D. Faller; Fig. 1B).

**Transfection and screening for cell lines with inducible T antigens.** The F-111 established line of rat embryo fibroblasts was used for all experiments (10, 13, 31). Plasmid DNAs were mixed in a ratio of 100 pSVM-mT (or pSVM-gen) to 1 pMoneo and transfected into F-111 cells by the calcium phosphate method of Graham and van der Eb (14). Cells were then selected for resistance to G418 in the absence of dexamethasone. Individual colonies were picked without regard to morphology. These were then propagated in the presence and absence of 1 μM dexamethasone and screened for levels of mT-associated kinase in a standard immunoprecipitation assay (28). This assay measures phosphorylation of mT on tyrosine, catalyzed by pp60$^{c\text{-}src}$ (5). The uninduced and induced levels of mT varied from clone to clone, a result which is consistent with previous observations with MMTV (9; Fig. 2A). Of 158 pSVM-mT-transfected lines, only 9 showed inducible levels of mT-associated kinase higher than 10% of the level of typical wild-type polyomavirus-

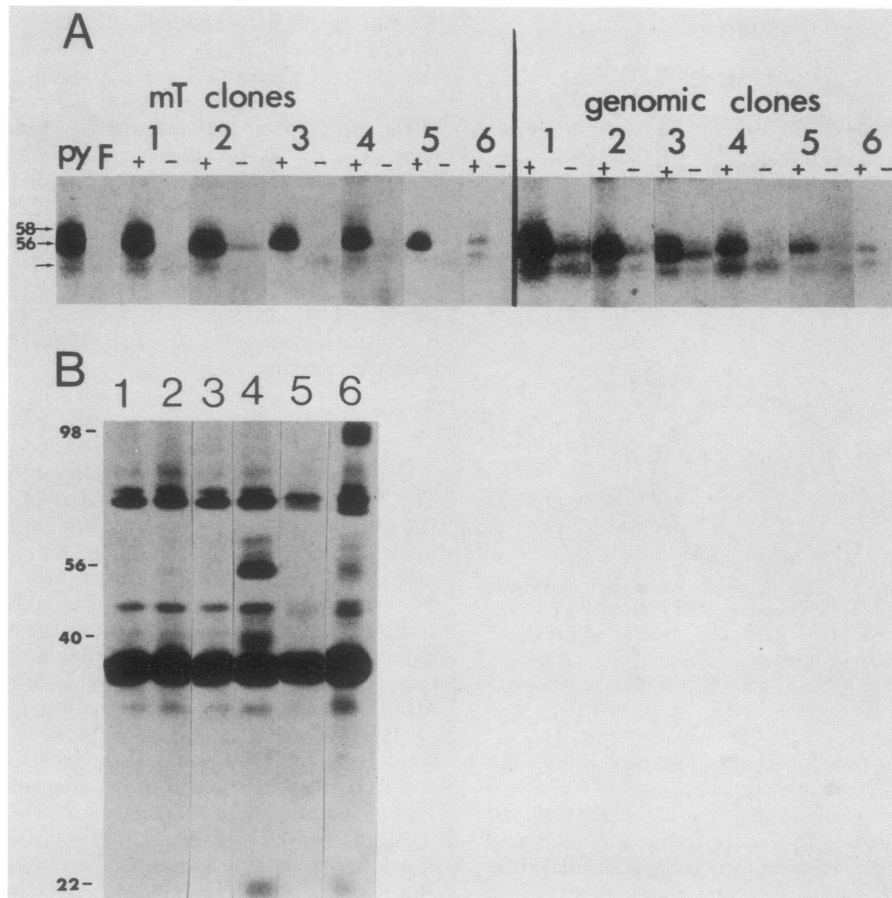


FIG. 2. (A) In vitro phosphorylation of mT produced in various inducible clones in the presence (+) or absence (−) of the inducer (1 μM dexamethasone). Washed immunoprecipitates were incubated with [$\gamma$-$^{32}$P]ATP, washed, and electrophoresed on 10% acrylamide-$NaDodSO_4$ gels. Gels were treated with 1 M NaOH (60 min, 60°C), dried, and exposed to X-ray film. Py, polyomavirus-transformed rat F-111 cells; F, the parental F-111 line. Arrows point to the positions of the 56K and 58K mT-associated kinase bands and to a 54K diffuse cellular band. The levels of mT phosphorylation in the presence and absence of dexamethasone were determined by densitometry. The values (+/− dexamethasone) were as follows, expressed as percentage of the level in pyF-111: mT-1, 100/<1%; mT-2, 80/1%; mT-3, 30/<1%; mT-4, 26/<1%; mT-5, 14/<1%; mT-6, 2/<1%; gen-1, 100/2%; gen-2, 36/1%; gen-3, 30/2%; gen-4, 22/<1%; gen-5, 3/<1%; gen-6, 1/<1%. (B) T antigens of inducible clones mT-1 and gen-1 shown in panel A labeled in vivo with [$^{35}$S]methionine (1 mCi/ml) for 8 h in the presence or absence of the inducer. Washed immunoprecipitates were electrophoresed on 10% acrylamide-$NaDodSO_4$ gels. Lanes: 1 and 2, clone mT-1 without and with dexamethasone, respectively; 3 and 4, clone gen-1 without and with dexamethasone; 5, rat F-111 cells; 6, polyoma virus-transformed rat F-111 cells.

transformed F-111 cells, whereas the corresponding numbers for the pSVM-gen transfectants were 122 and 8, respectively. Incubating cells with dexamethasone before and during the first 24 h after transfection had no effect on the frequency of inducible clones. Among the most inducible clones were mT-1 and gen-1, which showed induction ratios of 50 to 100. Uninduced levels in these clones were less than 2%, and fully induced levels were roughly 100% that of typical polyomavirus-transformed F-111 cells.

Eight-hour labeling of mT-1 and gen-1 cells with [$^{35}$S]methionine was done to estimate steady-state levels of T antigens. Levels of mT and small T antigen (sT) in stably transformed cells are usually low and frequently difficult to detect by metabolic labeling. This is shown in Fig. 2B, wherein F-111 (lane 5) is compared with pyF-111 (lane 6); weak labeling of mT and sT is seen in PyF-111. With gen-1, however, sT and mT are clearly labeled in response to dexamethasone, along with the expected large T fragment at the ~40,000-molecular-weight marker (lanes 3 and 4). MT-1, like pyF-111, showed minimal discernible labeling of mT in the presence of inducer. Gen-1 (lane 4) produced 100-fold higher levels of mT when fully induced than mT-1 (lane 2), despite the fact that the two clones showed similar levels of mT measured as a substrate in the immune complex kinase assay (Fig. 2A). MT-1 was similar to most of the inducible clones tested and to pyF-111 cells in its low overall level of mT and high specific activity of kinase active mT. Four of the inducible clones examined (three with the genomic plasmid and one with mT-cDNA) resembled gen-1 in showing disproportionately large amounts of mT by metabolic labeling compared with their levels measured in the in vitro kinase assay.

**Quantitation of mT-associated kinase activity in inducible clones.** Phosphorylation of mT in immunoprecipitates is stable (8) and can therefore be used to quantitate mT present in precipitated enzyme-substrate complexes. This assay was

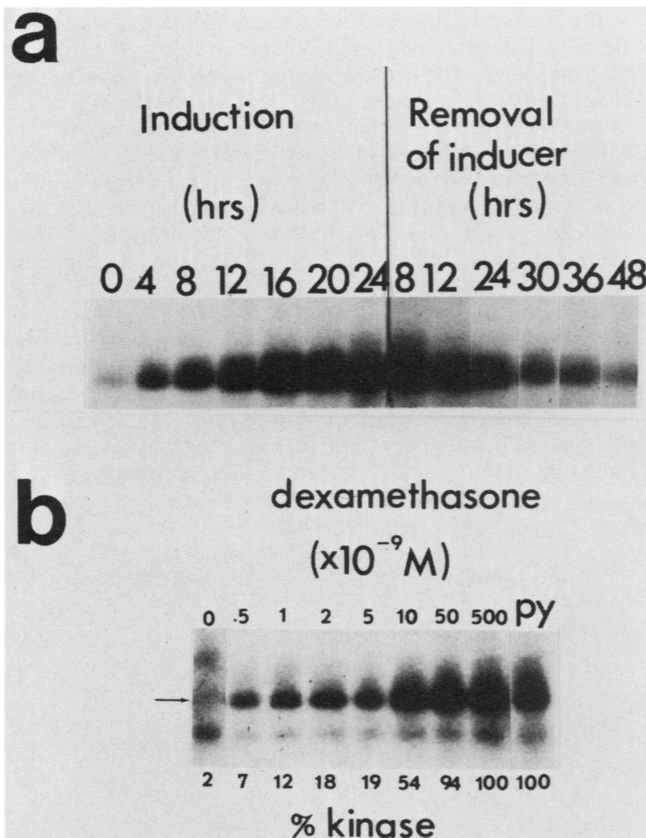


FIG 3. Time and inducer concentration dependence of the mT-associated kinase activity. (A) At the indicated times after the addition of 1 μM dexamethasone to clone gen-1, T antigens were extracted, phosphorylated in vitro with [γ-$^{32}$P]ATP, and electrophoresed on 10% acrylamide-sodium dodecyl sulfate gels. Similarly, after the addition of 1 μM dexamethasone for 24 h to achieve a full induction, the inducer was removed for the number of hours indicated before T antigen extraction, in vitro phosphorylation with [γ-$^{32}$P]ATP, and electrophoresis. (B) Clone mT-1 was grown in the presence of the indicated concentrations of dexamethasone for 48 h before T antigen extraction, in vitro phosphorylation with [γ-$^{32}$P]ATP, and electrophoresis. The intensity of the band indicated by the arrow, representing the 56K mT-associated kinase activity, was calculated by densitometry of the film (see the text), and the numbers obtained, expressed as a percentage of the corresponding number of a polyomavirus-transformed F-111 line (Py), are indicated.

used to follow the kinetics of induction and dose response with dexamethasone. Preliminary experiments were performed to assure that immune precipitations were carried out in antibody excess and that incubation times with [γ-$^{32}$P]ATP were such as to allow maximal levels of phosphorylation of mT. Sufficient cold ATP was present to assure steady labeling conditions throughout the incubation (see above).

The immune complex kinase assay was first used to follow the kinetics of appearance of mT after the addition of maximal levels of dexamethasone (1 μM). The steady-state level of mT was attained within about 16 h after the addition of the hormone, whereas at 4 to 8 h the level was approximately twice that of uninduced cells. Conversely, when dexamethasone was removed, the background level was reached in about two days (Fig. 3A). RNA dot blot experiments (39) showed increases in polyoma-specific cytoplasmic RNA as early as 30 min and maximum levels at approximately three h after induction (data not shown). The gen-1 clone showed high-level RNA expression compared with mT-1 and pyF-111, indicating that the overproduction of T antigens in this clone is determined, at least in part, at the transcriptional level. These kinetic parameters are consistent with those reported previously in studies with the MMTV-LTR promoter (18, 25).

A titration of in vitro phosphorylated mT in immune complexes as a function of dexamethasone concentration was also performed on several of the inducible clones. Representative results are shown in Fig. 3B. Concentrations of dexamethasone as low as $5 \times 10^{-8}$ M gave near maximal levels of induction. Fifty percent maximal induction occurred between $5 \times 10^{-9}$ and $10^{-8}$ M dexamethasone, in agreement with the reported binding constant for the glucocorticoid receptor (15), indicating that the increase in mT expression is in fact mediated by the known cytoplasmic receptor.

**Hormonal regulation of the transformed phenotype.** With several of the most responsive clones, quantitative control over levels of mT expression could be achieved over a broad range by varying the amount of hormone in the medium. The dependence of various parameters of transformation on the levels of mT-associated kinase could thus be followed.

**(i) Anchorage-independent growth requires maximal levels of mT expression.** Growth in soft agar was clearly dependent on mT expression (Fig. 4). All clones with induced levels of mT-associated kinase comparable to that in polyomavirus-transformed cells grew as efficiently in semisolid nutrient agar containing 2% calf serum as did the pyF-111 cells themselves. In contrast, clones in which the maximal induced levels were 20% or less than that of pyF-111 (e.g., mT-5, mT-6, gen-5, or gen-6; Fig. 2) failed to grow in soft agar even when fully induced. No differences between mT and genomic clones were noted in these experiments. This suggests that it is the level of mT expressed in kinase-active complexes that is important for anchorage-independent growth and that the presence of the sT protein and large T fragment in addition to mT has little or no effect.

The inducible clone mT-1 was used to examine this dependence further. Titrations of growth in soft agar in the presence of various concentrations of dexamethasone are shown in Fig. 4. Some growth was seen when mT was induced to ~20% maximal level ($5 \times 10^{-9}$ M dexamethasone), and the growth response increased roughly proportionally with the amount of dexamethasone up to that giving 100% induction of mT. A similar dose-response relationship was seen when the serum concentration in the agar was lowered from 2 to 0.4%. The growth of pyF-111 cells was roughly the same in the presence or absence of hormone.

A more quantitative estimate of anchorage-independent growth could be achieved by following incorporation of [$^3$H]thymidine into trichloroacetic acid-insoluble material in mT-1 and gen-1 cells suspended in methocel culture. Table 1 shows data relating thymidine incorporation to dexamethasone concentration and mT expression. The results show a graded response, with effects seen down to the lowest level of dexamethasone tested ($2 \times 10^{-9}$ M) which gives 15% maximal mT induction.

**(ii) Focus formation has a lower requirement for mT expression than growth in soft agar.** The ability of cells of the

inducible lines to grow as dense foci in monolayers of normal F-111 cells was studied as a function of dexamethasone concentration. Focus formation was clearly regulated by the hormone, as shown by the experiment in Fig. 5 with mT-1 cells. Approximately 200 mT-1 cells were plated along with $2 \times 10^4$ normal F-111 cells into 60-mm dishes in medium containing 5% calf serum. When these cultures were allowed to grow out in the presence of dexamethasone at $5 \times 10^{-10}$ M, a number of foci were clearly discernible (Fig. 5B). The measured amount of mT-associated kinase at this hormone concentration was 7% of the maximum inducible level. At higher concentrations, the number of foci increased, with a maximum response occurring at $5 \times 10^{-9}$ M which corresponds to ~20% maximal kinase activity. Still higher hormone concentrations increased the amount of mT-associated kinase activity without affecting the number or morphology of foci. Similar results were obtained with three other clones, including two genomic clones. Cells of the mT-1 clone also formed foci in a hormone-dependent manner when plated directly on top of preformed confluent monolayers of F-111 cells.

The dose-response curve for focus formation was clearly shifted to lower levels of hormone and of mT expression than the curve for growth in soft agar. This can be seen clearly from the response of mT-1 cells growing at $5 \times 10^{-9}$ M dexamethasone, which gives about 20% maximal mT expression in the kinase assay. Under these conditions, mT-1 cells fully expressed their potential to grow as dense foci in monolayers (Fig. 5B) but were barely able to grow in soft agar (Fig. 4B). When the concentration of dexamethasone was lowered to $2 \times 10^{-9}$ M, no clones appeared in soft agar, although focus formation was about 70% of the maximum value.

**(iii) Morphological transformation is the parameter most sensitive to mT expression.** Focus formation is frequently a composite property with a morphological as well as a growth differential component. To separate out and examine morphological changes per se, we plated cells from several of the inducible lines in the presence and absence of dexa-

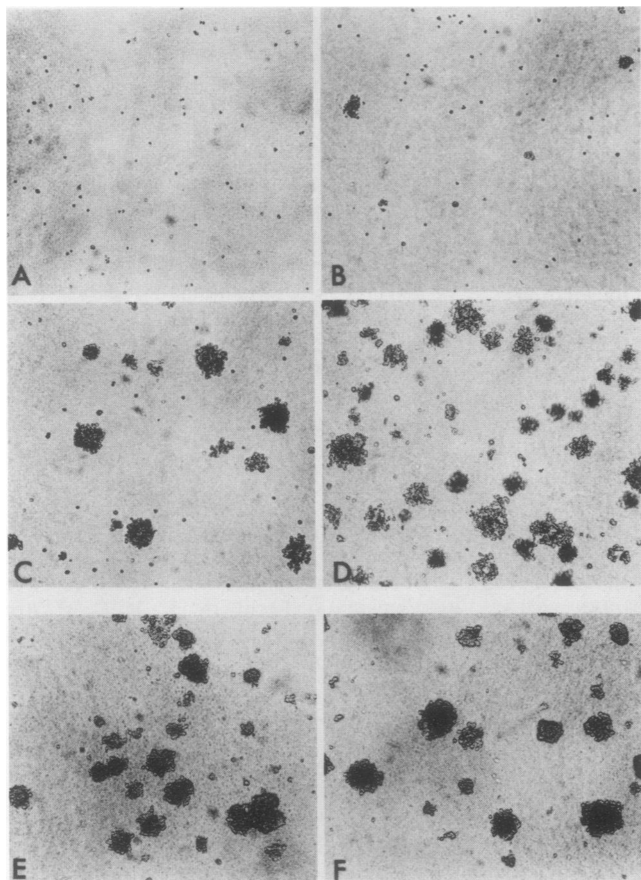


FIG. 4. Agar suspension assay of clone mT-1 in the presence of increasing dexamethasone concentrations. (A) $0.5 \times 10^{-9}$ M dexamethasone (kinase activity, 7% of the pyF-111 line, Fig. 3B). (B) $5 \times 10^{-9}$ M dexamethasone (kinase activity, 19%). (C) $50 \times 10^{-9}$ M dexamethasone (kinase activity, 94%). (D) $500 \times 10^{-9}$ M dexamethasone (kinase activity, 100%). (E and F) Polyomavirus-transformed F-111 cells, without and with 1 μM dexamethasone, respectively.

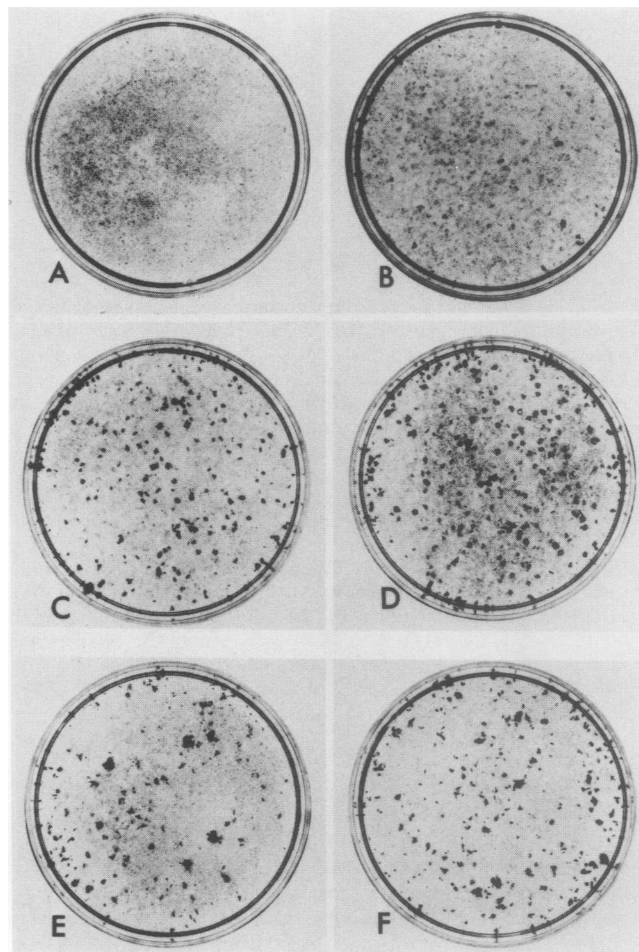


FIG. 5. Transformed cell focus assay of clone mT-1. Cells were seeded together with a hundredfold excess of F-111 cells in the presence of increasing dexamethasone concentrations (see the text). (A) No dexamethasone. (B) $0.5 \times 10^{-9}$ M dexamethasone (kinase activity, 7% of the pyF-111 line, Fig. 3B). (C) $1 \times 10^{-9}$ M dexamethasone (kinase activity, 12%). (D) $5 \times 10^{-9}$ M dexamethasone (kinase activity, 19%). (E and F) Polyomavirus-transformed F-111 cells, without and with 1 μM dexamethasone, respectively.

TABLE 1. Relationship between thymidine incorporation and dexamethasone concentration and mT expression

| Clone | Dexamethasone (M)[a] | Thymidine incorporation (cpm)[b] | % cpm[c] | Kinase activity (%)[d] |
|---|---|---|---|---|
| mT-1 | 0 | 151 | 0.7 | 2 |
| | $2 \times 10^{-9}$ | 1,100 | 5.0 | 18 |
| | $10^{-8}$ | 10,052 | 45 | 54 |
| | $5 \times 10^{-8}$ | 18,686 | 84 | 94 |
| | $10^{-6}$ | 22,150 | 100 | 100 |
| gen-1 | 0 | 258 | 2 | 2 |
| | $2 \times 10^{-9}$ | 1,898 | 8 | 15 |
| | $10^{-8}$ | 8,560 | 55 | 61 |
| | $5 \times 10^{-8}$ | 12,238 | 80 | 92 |
| | $10^{-6}$ | 15,386 | 100 | 100 |
| pyF-111 | 0 | 25,879 | 93 | 100 |
| | $10^{-6}$ | 27,817 | 100 | 100 |
| F-111 | 0 | 269 | 94 | |
| | $10^{-6}$ | 286 | 100 | |

[a] Cells of the mT-1 and gen-1 clones were suspended in methocel containing 10 μCi of [$^3$H]thymidine per ml and various concentrations of dexamethasone as indicated.

[b] After 20 days of suspension, the cells were washed from the methocel, and trichloroacetic acid-precipitable counts were determined.

[c] Incorporation relative to that in 1 μM dexamethasone.

[d] Kinase activity of the cells growing in liquid medium relative to that in 1 μM dexamethasone.

methasone. Inducible clones in which the background levels of mT expression were even a few percent that of pyF-111 cells showed morphological changes in the absence of hormone when compared with normal F-111 cells; furthermore, these clones did not change in appearance dramatically in the presence of 1 μM dexamethasone. Only the clones expressing the lowest background of mT-associated kinase (<1% of pyF-111 cells) retained normal or near-normal morphology. Such lines could be shown to undergo discernible morphological transformation in the presence of dexamethasone. Clone mT-6 in the absence of hormone closely resembled normal F-111 cells. Individual cells were largely bipolar and showed parallel alignment with their neighbors. When fully induced, these cells expressed only 2 to 3% of the level of mT-associated kinase activity of pyF-111 cells (Fig. 2A) and yet underwent clear morphological transformation (Fig. 6). The induced cells became more polygonal, overlapped with their neighbors, and tended toward a multilayered growth pattern akin to that of pyF-111 cells. Normal F-111 cells and pyF-111 cells showed essentially no change in morphology or growth pattern in response to dexamethasone.

**(iv) Dose-response curves emphasize the importance of the mT-associated kinase activity.** Figure 7 presents the combined dose-response data for different parameters of transformation. The order of emergence of the three parameters under study as a function of the amount of mT-associated kinase is clear. In the case of growth in soft agar, the dependence on level of induction is seen over the broadest

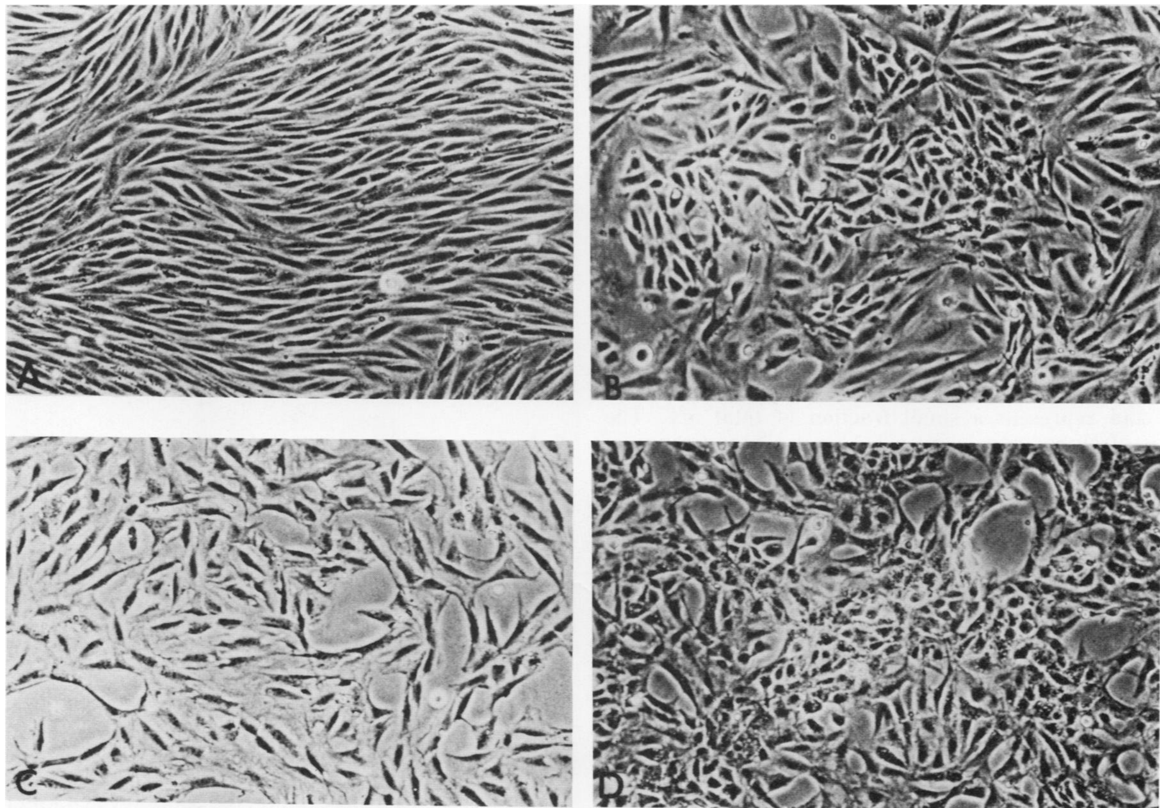


FIG. 6. Morphological alterations of inducible lines after the addition of dexamethasone. (A and B) Clone mT-6 in the absence and presence of 1 μM dexamethasone, respectively. (C and D) Clone mT-5 in the absence and presence of 1 μM dexamethasone, respectively.

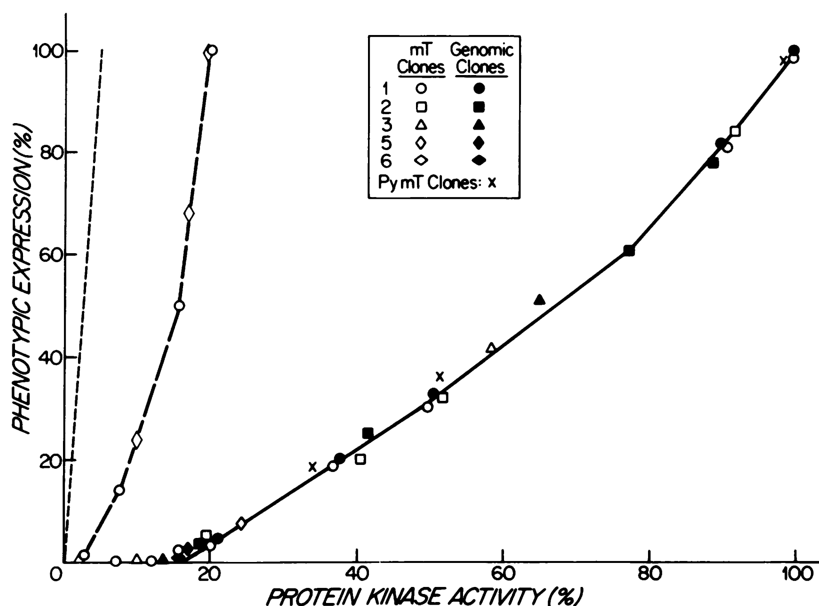


FIG. 7. Dependence of phenotypic expression on kinase activity. Solid line, growth in methocel (determined as described in footnote *a* to Table 1); heavy broken line, foci formation; dashed line, morphology.

range. The points on this curve as well as that for focus formation show remarkably little scatter. They represent inducible clones expressing either mT alone or mT along with sT and the large T fragment. Noninducible clones obtained by transfection with mT cDNA and expressing fixed levels of mT independent of hormone also fall on the same curve. These results thus serve to underline the biological importance of the kinase-active fraction of mT in transformation.

**(v) Reversal of the transformed phenotype upon removal of dexamethasone.** Several mT and genomic clones were fully induced by growth in the presence of 1 μM dexamethasone for 24 h and then washed and suspended in soft agar in the absence of hormone. All failed to grow despite having high initial levels of mT-associated kinase. Together with the kinase data of Fig. 3A showing decay to background levels within 2 days after the removal of hormone, these results indicate a continuous requirement for high-level expression of mT to sustain anchorage-independent growth.

**mT molecules active in transformation are complexed with pp60$^{c\text{-}src}$ and represent a small fraction of total mT.** The responses of clones gen-1 and mT-1 over a broad range of dexamethasone concentrations were virtually identical, both in phenotype (Fig. 7) and in terms of the amounts of mT-associated kinase activity in anti-T immune complexes (Fig. 2A). It was therefore surprising to find gen-1 producing 100-fold more mT than mT-1 by [$^{35}$S]methionine labeling (Fig. 2B). The amount of mT in fully induced gen-1 cells was in fact considerably more than that found in typical wild-type virus-transformed F-111 cells.

The gen-1 clone was therefore examined further by using anti-*src* and anti-T antibodies in direct and sequential immunoprecipitation experiments (Fig. 8). Extracts of [$^{35}$S]methionine-labeled gen-1 cells grown for 24 h in the presence of 1 μM dexamethasone were precipitated with either anti-T serum or anti-*src* monoclonal antibody 327 (kindly provided by Joan Brugge). The supernatant of the anti-*src* precipitation was then reprecipitated with anti-T serum and run alongside the immunoprecipitate made directly with anti-T. Roughly equal amounts of [$^{35}$S]methionine-labeled mT were brought down (Fig. 8, lanes 1 and 2), indicating that the anti-*src* serum had removed only a very small amount of the

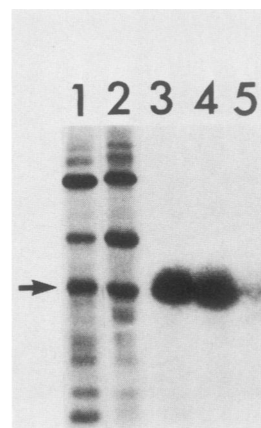


FIG. 8. Immunoprecipitation profiles of [$^{35}$S]methionine-labeled T antigens from clone gen-1 grown in the presence of 1 μM dexamethasone. The T antigen extract was divided into several aliquots. Lane 1, Aliquot precipitated with anti-T ascites fluid. Lane 2, Another aliquot of the same extract absorbed out with anti c-*src* monoclonal antibody 327. The supernatant was then precipitated with anti-T ascites fluid. Lane 3, In vitro [γ-$^{32}$P]ATP phosphorylation of another aliquot after immunoprecipitation with anti-T ascites fluid. Lane 4, Same as in lane 3; immunoprecipitated with the same amount of 327 anti c-*src* monoclonal antibody. Lane 5, After the extract was absorbed out with the anti c-*src* monoclonal antibody, the supernatant was precipitated with anti-T ascites fluid as in lane 2 above. The washed immunoprecipitate was subsequently labeled with [γ-$^{32}$P]ATP. In lanes 1 and 2, the gel was treated with PPO (2,5-diphenyloxazole)-dimethyl sulfoxide and exposed without an intensifier screen. In lanes 3 to 5, the gel was treated with 1 M NaOH and exposed with an intensifier screen. Arrow points to the 56K mT protein.

TABLE 2. Tumorigenicity of inducible clones

| Clone | Dexa-methasone[a] | Kinase[b] | No. of animals with tumors/ no. of animals injected[c] |
|---|---|---|---|
| mT-1 | + | 100 | 8/8 |
| | − | 2 | 3/4 |
| gen-1 | + | 100 | 5/5 |
| mT-3 | + | 50 | 3/5 |
| mT-5 | + | 18 | 1/5 |
| mT-6 | + | 3 | 0/4 |

[a] A total of 0.02 mg per rat per day given in the food.

[b] Kinase values represent maximum inducible levels of the cells in culture ($10^{-6}$ M dexamethasone) expressed as a percentage of pyF-111 cells.

[c] Tumor appearance was scored 30 days after subcutaneous injection of approximately $10^6$ cells into 2-week-old male Fisher rats. Cells were grown in the presence of $10^{-6}$ M dexamethasone for 24 h before inoculation.

total metabolically labeled mT protein. Lanes 3, 4, and 5 show results of kinase reactions run in parallel on immunoprecipitates prepared in the same fashion. Direct precipitation with anti-T (lane 3) or anti-*src* (lane 4) brought down equivalent amounts of mT substrate for pp60$^{c\text{-}src}$ kinase. When the supernatant of the anti-*src* precipitation was reprecipitated with anti-T serum and incubated with [$\gamma$-$^{32}$P]ATP, only a trace of phosphorylatable mT was brought down (lane 6). Thus, anti-*src* serum effectively removed most of the mT molecules capable of being phosphorylated by pp60$^{c\text{-}src}$, while leaving behind the bulk of mT protein.

These results show that the various parameters of transformation reflect the kinase-active fraction of mT, i.e., the portion bound to pp60$^{c\text{-}src}$, and not the overall level of mT protein in the cell. Similar experiments by Bolen et al. (3) with anti-T first to clear extracts of mT followed by anti-*src* (the same monoclonal as used here), showed that the bulk of pp60$^{c\text{-}src}$ is not complexed with mT. Thus, both components of the complex exist "free" and in apparent excess when complexes are formed. We suggest that cellular mechanisms, possibly involving additional kinases, in some way regulate the formation of active complexes between these two proteins (see below).

**The ability to form tumors requires maximal inducible levels of mT-associated kinase.** Young male Fisher rats were inoculated subcutaneously with cells from various clones expressing different maximal inducible levels of mT-associated kinase activity. The fraction of animals with palpable tumors after 30 days increased from 0 to 100% (Table 2), roughly in parallel with the kinase levels expressed in culture when the cells are fully induced. Cells from several tumors were put back in culture; these tumor-derived lines were indistinguishable from the original cells in terms of inducibility.

Although the correspondence between tumorigenicity and mT inducibility was seen in animals fed daily with dexamethasone, the addition of hormone to the feed was not essential. In the group of animals inoculated with mT-1 cells and maintained without dexamethasone, three out of four animals developed tumors. As found by Jakobovits et al. (17) for pp60$^{v\text{-}src}$ under MMTV-LTR promoter control, endogenous glucocorticoid levels can be sufficient for near maximal levels of mT induction, because the expected level of mT expression by mT-1 cells in the absence of dexamethasone is only 2%. This level is similar to that of fully induced mT-6 cells which failed to cause tumors in dexamethasone-fed rats. Immunosuppressive effects of dexamethasone were expected with the doses used here. However, such effects do not obscure the determining role of mT inducibility as the controlling factor in tumor formation.

In another experiment, animals with mT-1-induced tumors that were maintained on dexamethasone showed complete regression of palpable tumors 3 weeks after the withdrawal of hormone from the diet. In this case, endogenous steroids were probably suppressed below the level required to sustain tumor growth. Reintroduction of dexamethasone to the diet led to the reappearance of tumors. These results support the conclusion that growth of inducible cells in the animal as a tumor is regulated by steroids and the maximal levels of mT expression are required for tumor formation. These levels were in the range required for growth in soft agar, in agreement with earlier results correlating anchorage-independent growth with tumor formation in nude mice (12).

## DISCUSSION

Early attempts to relate the action of viral oncogenes to particular features of neoplastically transformed cells were frustrated by a variety of factors—notably, heterogeneity of expression of transformation parameters even with cloned established cells as targets (24) and the lack of assays to measure levels of viral oncogene expression in individual clonal isolates of transformed cells. Temperature-sensitive mutants of Rous sarcoma virus partially defective in transformation have been isolated and characterized in their interactions with chicken embryo primary cultures (1). With some of these mutants, a clear dissociation occurs between focus formation which was temperature sensitive and growth in soft agar which was not; surprisingly, based on the hierarchy shown here for polyoma and rat fibroblasts, these mutants were nontumorigenic. A more recent approach which bypasses these problems utilizes transfection with plasmids expressing oncogenes whose products can be assayed and whose expression is regulated by a suitable heterologous promoter. Such approaches have been reported with H-*ras* (15, 16), v-*mos* (20), and v-*src* (17), all under regulation of the dexamethasone-inducible promoter (MMTV-LTR). Thresholds of viral gene activity for particular cellular responses have been demonstrated in some cases, although the range of induction and variety of cell responses that have been studied are somewhat limited.

We isolated and studied a series of clones of established rat fibroblasts in which the dose of polyoma mT antigen can be regulated over a 100-fold range. Different transformation parameters, previously taken as indicative of neoplastic transformation, all showed dependence on the level of mT induction but evolved over different ranges. Morphological transformation, growth as foci on normal cell monolayers, and anchorage-independent growth evolved progressively with increasing levels of mT expression. Titrations of more than a dozen different cell lines showed no exceptions to this hierarchy. Only those clones whose maximum inducible levels of mT were sufficiently high to promote growth in soft agar could form tumors.

Morphological aspects of transformation were the most sensitive to mT expression and occurred at levels only a few percent of that expressed in typical wild-type virus-transformed cells. These changes in turn have previously been correlated with loss of actin cables (31) and of extracellular fibronectin (19). Focus formation, which reflects loss of density regulation of growth as well as morphological

change, requires up to 20% maximal levels of mT for full expression. Anchorage-independent growth exhibits quantitative dependence over the broadest range of inducer concentration, beginning at about 15 to 20% and increasing roughly in proportion to hormone concentration up to that giving 100% mT expression.

Formation of tumors in the syngeneic host depends on the maximum level of mT inducibility attainable by the clone in culture and reflects most closely the range required for growth in soft agar. Clearly, in this virus-host system, neither morphological transformation nor focus formation by itself is a reliable predictor of tumorigenic transformation. Levels of mT sufficient to give tumorigenicity are higher by one or two orders of magnitude than those required for morphological transformation and three- to fivefold higher than that which gives efficient focus formation. Endogenous glucocorticoid levels in the intact host are sufficient to give maximal or near maximal induction in injected cells. However, prior administration of dexamethasone can apparently suppress adrenal function sufficiently so that tumor growth becomes dependent on exogenous steroids.

Clones of F-111 cells were also isolated after transfection with a plasmid encoding an mT cDNA with the normal polyoma promoter (37) and coselection with neo. Levels of mT in these clones varied from 20 to 100% of mT levels in wild-type virus-tranformed cells. These hormone-independent mT transformants fell on the same dose-response curve as that defined by the inducible clones (Fig. 7). This reinforces the conclusion that mT as measured in the in vitro kinase assay is the determining factor in the degree of biological response and suggests that the general effects of dexamethasone per se do not contribute significantly to those responses. Because dexamethasone by itself induces clumping of cells in suspension (5a), the phenomenon of agglutinability by lectins could not be studied in the inducible clones. However, the hormone-independent mT transformant expressing the lowest level of in vitro kinase (20%) was found to be fully agglutinable by concanavalin A.

Clones expressing mT alone responded in the same way as those expressing the subgenomic early fragment which encodes sT and an amino terminal large T fragment in addition to mT. In both types of clones, the level of mT expressed in the immune complex kinase assay appeared to determine the degree of phenotypic expression in transformation. These results, while emphasizing the importance of mT-associated kinase activity, do not imply a lack of biological activity of the other T antigens that might be evident, for example, in the absence of mT or in other cell systems. A virus mutant totally devoid of mT expression but encoding both small and large T antigens was recently shown to possess mitogenic activity and to induce lectin agglutinability (19), and studies with sT cDNA have indicated an activity of the sT protein in inducing loss of cell-substratum adhesion (6).

Polyomavirus-transformed F-111 cells typically express low levels of mT protein that are difficult to detect by steady-state metabolic labeling with [$^{35}$S]methionine (Fig. 2B, lane 6). Most of the transfectants isolated in this study showed similar low levels of mT. However, several inducible clones, such as gen-1, expressed much higher than normal levels of mT after induction by the hormone (Fig. 2B, lane 4). Interestingly, these high producer clones did not express correspondingly high levels of mT as measured in the immune complex kinase assay. The kinase-active mT complexes from this line were immunoprecipitated with equal efficiency by anti-T and anti-*src* antibody, whereas the bulk of [$^{35}$S]methionine-labeled mT was efficiently precipitated only by anti-T sera (Fig. 8). Gen-1 cells, and others like them, had the same dose-response behavior as other inducible lines. Clearly, it is not the overall level of mT present in the cells after induction which correlates with transformation parameters and tumorigenicity but rather the variable and usually small fraction of mT which can be isolated in a complex with pp60$^{c\text{-}src}$. These results reinforce the earlier work of Courtneidge and Smith (5) first demonstrating mT-pp60$^{c\text{-}src}$ complexes and the more recent work of Bolen et al. (3) showing enhancement of tyrosyl protein kinase activity of pp60$^{c\text{-}src}$ when complexed with mT.

The mT molecules active in immune complexes as a substrate for pp60$^{c\text{-}src}$ behave differently from the bulk of metabolically labeled mT in their more rapid sedimentation profile (28) and in their preferential association with Triton-resistant cell frameworks (29, 32). This same fraction of active mT molecules can also be distinguished by metabolic labeling with [$^{32}$P]orthophosphate. The 56K and 58K forms of mT, labeled in vivo with $^{32}$P at different sites by cellular serine kinases (26), are also preferentially framework bound (29) and can be efficiently precipitated from lytically infected cell extracts with anti-*src* as well as anti-T antibodies. We have recently obtained evidence that protein kinase C is involved in the phosphorylation of the 58K form of mT in vivo (J. Matthews and T. Benjamin, manuscript submitted). Previous studies of *hr-t* and other mutants defective in mT have linked in vivo phosphorylation of 58K mT with acquisition of in vitro kinase activity and transforming ability (4, 5, 27). To determine whether the in vivo phosphorylated forms of mT in the inducible clones correspond to the kinase-active fraction measured in vitro, we performed labeling of mT-1 and gen-1 cells with [$^{32}$P]orthophosphate in the presence of dexamethasone. Roughly equal although very low amounts of incorporation were detected, a result which parallels the similar levels of in vitro kinase-active mT and not the vastly different total amounts of mT present in these two lines.

The precise mechanisms that regulate the formation of active mT:pp60$^{c\text{-}src}$ complexes from the available pools are unclear. The mT protein itself lacks ATP-binding activity (29) and possesses no intrinsic kinase activity when expressed in *E. coli* (28a). Interaction with the cellular tyrosine kinase pp60$^{c\text{-}src}$ appears to be essential. The serine kinases which phosphorylate mT in vivo may activate the latter to promote its interaction with pp60$^{c\text{-}src}$ or act on the mT:pp60$^{c\text{-}src}$ complex to alter its function. The requirements for postsynthetic modification of mT and its interaction with multiple cellular kinases probably accounts, at least in part, for the delay between induction of mT gene transcription (~3 h) and the appearance of active mT:pp60$^{c\text{-}src}$ complexes in the plasma membrane (~16 h). Regardless of the mechanism of activation, the ability to titrate the biological responses in inducible clones as shown here should facilitate future work aimed at uncovering individual cellular targets or substrates of the activated mT:pp60$^{c\text{-}src}$ complexes that are involved in those responses.


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