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Cooperation of Middle and Small T Antigens of Polyomavirus in Transformation of Established Fibroblast and Epitheliallike Cell Lines

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We have reported recently that small T antigen of polyomavirus stimulates the growth of NIH 3T3 cells beyond their saturation density and induces weak anchorage-independent growth (T. Noda, M. Satake, T. Robins, and Y. Ito, J. Virol. 60:105–113, 1986). We examined whether small T antigen would cooperate with middle T antigen in the in vitro transformation of NIH 3T3 (fibroblasts) and NRK-52E (epitheliallike) cells. The small-T-antigen gene, when cotransfected with the middle-T-antigen gene, had no additional effect on the efficiency or size of dense foci formation induced by the middle-T-antigen gene on a monolayer of NIH 3T3 cells. However, the small-T-antigen gene dramatically increased the rate of growth of NIH 3T3 cells transformed by middle T antigen in semisolid medium. Introduction of the small-T-antigen gene into middle-T-antigen-transformed cells did not disturb the integrated middle-T gene, alter expression of the middle-T gene, or enhance middle-T-antigen-associated tyrosine protein kinase activity. For NRK-52E cells, the expression of middle T antigen alone resulted in small, slow-growing foci on a monolayer. These cells did not show anchorage-independent growth, despite the fact that middle-T-antigen-associated tyrosine protein kinase activity was clearly detected in these cells. NRK-52E cells expressing both middle and small T antigens formed faster growing foci on a monolayer than middle-T-antigen-expressing cells did and grew in semisolid medium, even when the amounts of middle T antigen and its associated kinase activities were lower than those of middle-T-antigen-expressing cells. We conclude that small T antigen cooperates with middle T antigen in the in vitro transformation of established cell lines of fibroblast and epitheliallike cells, that it does not share the middle-T-antigen function even though they are structurally related, and that it has a significantly more important role in the transformation of NRK-52E cells than that of NIH 3T3 cells.

The early region of polyomavirus is required for cell transformation by the virus. This early region contains two genetic loci, *tsA* (14) and *hr*-t (4), and encodes three early proteins which share the amino-terminal region of the proteins (39). The early genes of the virus are organized in an overlapping manner. The *tsA* locus is the structural gene for large T antigen. The *hr*-t mutations are located within the segment of viral DNA which is an intron for the large-T-antigen gene. This segment of viral DNA is a part of the structural genes for middle and small T antigens. Except for the carboxy-terminal four amino acid residues, the primary structure of small T antigen is identical to that of the amino-terminal half of middle T antigen (for a review see reference 20).

The functions of large and middle T antigens are quite different and contribute to oncogenic cell transformation by cooperating with each other (32). It has been suggested that the role of large T antigen in the transformation of primary rat embryo fibroblasts is to immortalize cells, enabling cells to grow indefinitely (1, 24, 33). Immortalization is considered one of the essential steps for oncogenic transformation.

Middle T antigen is a membrane-bound protein (21) and is primarily responsible for inducing the major phenotype of transformed cells (23, 31). The only biochemical function known to be associated with middle T antigen is the tyrosine-specific middle-T-antigen-phosphorylating activity in vitro (12, 36, 40). This tyrosine protein kinase activity is considered to be due to an associated cellular tyrosine kinase, a product of proto-oncogene c-*src* (9). Middle T antigen has been shown to modify and activate c-*src* kinase activity (5).

The carboxy-terminal half of middle T antigen is unique to this protein. The in vitro phosphorylation of middle T antigen on tyrosine by $p60^{c\text{-}src}$ and membrane anchorage, which appears to be essential for the binding of middle T antigen to $p60^{c\text{-}src}$, both occur within this domain. There are some mutants which have altered transformation functions and have deletions within this domain (17, 28). On the other hand, *hr*-t mutations located in the amino-terminal half of middle T antigen abolish the ability of middle T antigen to modify and activate $p60^{c\text{-}src}$ (5). How the changes in this first domain affect the function unique to middle T antigen is not known.

Regardless of whether the association of middle T antigen and $p60^{c\text{-}src}$ is important for oncogenic transformation, it is worth noting that only a subfraction of middle T antigen is associated with $p60^{c\text{-}src}$ (5, 37) and that the subfraction of middle T antigen associated with $p60^{c\text{-}src}$ is present in the plasma membrane (37). Whether the middle T antigen subfraction which is not present in the plasma membrane or the middle T antigen subfraction which is not associated with protein kinase activity has any function is not known.

Recently, it has been reported that small T antigen is mainly localized to the nuclei, stimulates the growth of monolayers of NIH 3T3 cells beyond their saturation density, and induces the growth of cells in soft agar, although the activity of the latter is very weak (7, 30). Since middle and small T antigens are structurally related and are collectively called *hr*-t gene products, the question arises as to whether the function of small T antigen is also manifested by

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middle T antigen. If middle T antigen expresses small-T function as well, middle and small T antigens should not complement.

It has been suggested that small T antigen is required for the in vitro transformation of rat embryo fibroblasts in conjunction with large and middle T antigens (10). On the other hand, tumorigenesis in newborn rats appears to require the cooperation of either large and middle T or middle and small T antigens (2, 3). Although these reports suggest that small T antigen plays an important role, more detailed studies are necessary to understand the role of small T antigen in cooperation in cell transformation and tumorigenesis.

Polyomavirus induces tumors of epithelial cell origin (18). Transformation studies using polyomavirus, however, have been almost exclusively concentrated on fibroblasts. Since it is important to study the transformation of epithelial cells, we should take advantage of the fact that this virus can be used for the studies of epithelial cell transformation. Towards that goal, we have begun to use an established rat cell line, NRK-52E, which is considered to have some properties of epithelial cells (11).

In the present studies, we addressed the following questions. (i) Does small T antigen cooperate with middle T antigen in the in vitro transformation of established rodent cell lines NIH 3T3 (fibroblast) and NRK-52E (epitheliallike cells)? (ii) If it does, how? (iii) Is there any difference in the effect of small T antigen on the cell transformation of NIH 3T3 cells and NRK-52E cells? The results indicated that small T antigen cooperated with middle T antigen in cell transformation, that it contributed to the process of cell transformation by an entirely different mechanism from that of middle T antigen, and that it has a more important role in the transformation of NRK-52E cells than that of NIH 3T3 cells. A preliminary account of the cooperative effect of small T antigen on the growth of NIH 3T3 cells transformed by middle T antigen has been given (22).

MATERIALS AND METHODS

Cell lines and culture conditions. NIH 3T3 cells were a gift from Wallace Rowe (National Institutes of Health, Bethesda, Md.). NRK-52E (11), a cell line with epithelial cell-like properties isolated from a normal rat kidney, was obtained from American Type Culture Collection (Rockville, Md.). These cells were maintained in Dulbecco modified Eagle medium supplemented with 10% calf serum (Colorado Serum Company, Colorado) in a humidified atmosphere of 10% CO_2 in air.

Transfection, *neo* selection, and cell cloning. NIH 3T3, NRK-52E, and NIH 3T3 cells expressing middle T antigen, MT3, were transfected with plasmids by a calcium phosphate precipitation technique (16, 45). Form I of plasmid DNA was used for transfection. To select cells expressing a bacterial neomycin resistance gene, transfected cultures were incubated in a culture medium containing 400 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml (15, 43). The cells were cloned by limiting dilution. In some cases, the cells were isolated from dense foci on a monolayer by using cloning rings and were used as quasi-cell clone. Clones were numbered cl-1, cl-2, etc., while quasiclones were named f-1, f-2, etc.

Plasmids. pGVST, a retrovirus-based shuttle vector which can express the polyomavirus small-T-antigen gene and the bacterial gene for neomycin resistance derived from Tn*5* (15, 43) has been described (30). pGV16 is the backbone construct of pGVST and can only express a neomycin resistance gene (35). Plasmids pPyMT1 and pPyST1 (44, 46), which contain the entire polyomavirus genome but lack the middle-T-antigen intron (pPyMT1) or the small-T-antigen intron (pPyST1) cloned at the *Bam*HI site in pAT153, were obtained from Robert Kamen (Genetics Institute, Cambridge, Mass.). pSV2*neo*, which can express a neomycin resistance gene under the control of the simian virus 40 early region promoter, was obtained from George Pavlakis (National Cancer Institute, Frederick Cancer Research Facility, Frederick, Md.).

Protein kinase assay. Tyrosine-specific protein kinase activity associated with middle T antigen was assayed as described previously (37) with minor modifications. Briefly, cell extracts obtained from 10^5 cells were subjected to immunoprecipitation with hamster anti-polyomavirus T-antigen serum (37). The immunoprecipitates were extensively washed with RIPA buffer (34) containing 10 mM NaCl (26) and incubated in 50 μl of kinase buffer (50 mM Tris hydrochloride, pH 7.5, 5 mM $MgCl_2$) containing 10 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) for 10 min at 30°C. After the reaction was terminated by adding a large excess of NET (150 mM NaCl, 50 mM EDTA, 50 mM Tris hydrochloride, pH 7.4, 0.02% sodium azide, 0.05% Nonidet P-40), the immunoprecipitates were washed extensively with RIPA buffer containing 10 mM NaCl, eluted from protein A immunoadsorbent, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Under these conditions, over 95% of the radioactive phosphate incorporated into middle T antigen was found on tyrosine (37).

Immunoprecipitation, SDS-PAGE, and fluorography. [^{35}S]methionine-labeled polyomavirus T antigens were immunoprecipitated and analyzed by SDS-PAGE and fluorography as described previously (20).

Southern blot analysis. Cellular DNA was analyzed by the method of Southern (42), using standard protocol (29). Briefly, high-molecular-weight cellular DNA was extracted by dissolving EDTA-dispersed cells with 0.5 M sodium perchloride and 0.3% SDS, followed by two cycles of extraction with an equal-volume mixture of phenol and chloroform. The aqueous phase, dialyzed against 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, was used for the restriction enzyme analysis. A 20-μg sample of DNA was digested completely with various restriction enzymes, separated by 0.6% agarose gel electrophoresis, and blotted onto nitrocellulose filters. Baked filters were hybridized at 42°C in a solution of 50% formamide, 5× Denhardt solution, 5× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate) buffer, 10% dextran sulfate, 50 mM Tris hydrochloride (pH 7.4), 0.1% SDS, and 100 μg of shared calf thymus DNA per ml. A restricted polyomavirus (A2 strain) DNA fragment from *Bam*HI to *Eco*RI (nucleotide [nt] 4632 through 1550) was nick translated and used as a probe. Hybridized filters were washed in 0.2× SSC buffer–0.1% SDS at 68°C and processed by autoradiography.

Northern blot analysis. Northern blot analysis was performed essentially as described (29). Briefly, total cellular RNA was isolated from cells by lysing in GTC solution (5.7 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine, 0.1 M 2-mercaptoethanol), followed by centrifugation through 5.7 M cesium chloride (8). The pelleted RNA fraction was phenol extracted and ethanol precipitated. Heat- and formamide-denatured total RNA was electrophoresed through 1.0% agarose gels containing 2.2 M formaldehyde in a morpholinepropanesulfonic acid (MOPS)-

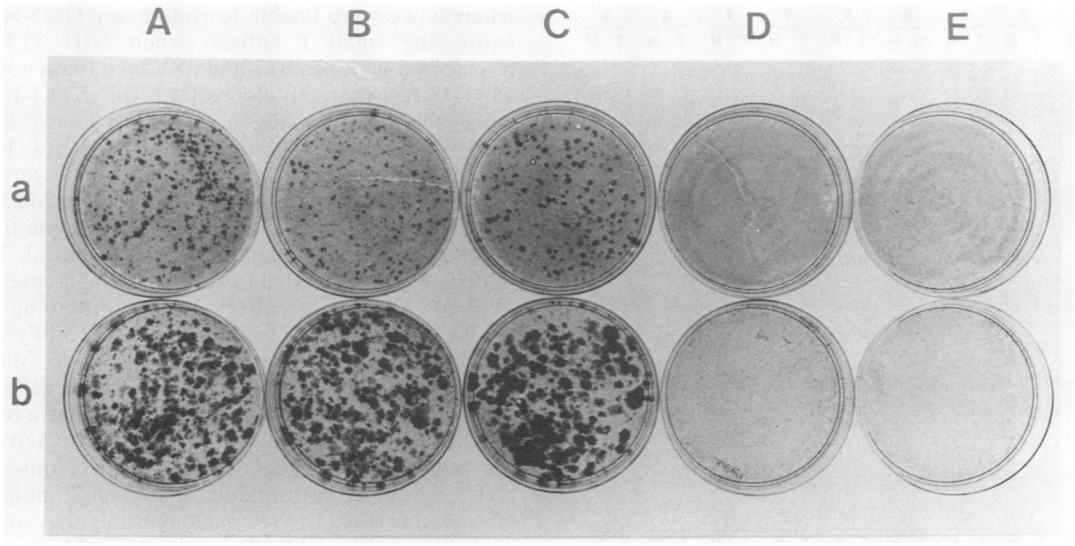

FIG. 1. Dense focus formation on monolayers of NIH 3T3 cells after transfection with pPyMT1, pPyST1, or mixture of the two. Each 100-mm dish was seeded with 10^5 NIH 3T3 cells 1 day before being transfected with pPyMT1 (1 μg per dish) (A), pPyMT1 (1 μg) and pPyST1 (1 μg) (B), pPyMT1 (1 μg) and pPyST1 (10 μg) (C), pPyST1 (10 μg) (D), or salmon sperm DNA (E). In all cases, the amount of total DNA transfected was adjusted to 20 μg with salmon sperm DNA. Cells were incubated in Dulbecco modified Eagle medium containing 5% calf serum for 2 weeks (a) or 3 weeks (b) and stained with Giemsa solution after fixation with a 1-to-1 mixture of methanol and acetone.

acetate buffer for 12 h at 25 V. The gels were blotted onto a nitrocellulose filter and baked in a vacuum oven at 80°C for 2 h. Nick-translated DNA fragments were hybridized to the filters in the same solution as the one used in Southern blotting, together with 100 μg of yeast tRNA. Filters were washed in 2× SSC–0.1% SDS at 65°C for 1 h and processed for autoradiography. A set of two identical gels were electrophoresed in parallel, one of which was stained with ethidium bromide to compare the amounts of RNA present in parallel lanes.

RESULTS

Cotransfection of pPyMT1 and pPyST1 into NIH 3T3 cells. To see how middle and small T antigens cooperate in the in vitro transformation of NIH 3T3 cells, monolayers of NIH 3T3 cells were transfected with pPyMT1, pPyST1, or a mixture of the two, and the cultures were fixed and stained after 2 or 3 weeks of incubation (Fig. 1). pPyMT1 induced numerous foci (Fig. 1A), while a 10-times-higher amount of pPyST1 did not induce any recognizable foci (Fig. 1D). The mixture of pPyMT1 and pPyST1 at a 1-to-1 (Fig. 1B) or 1-to-10 ratio (Fig. 1C) did not significantly alter the number or size of the foci which appeared.

However, when several foci were randomly picked from each of these dishes and the cells were suspended in soft agar (27), we observed that the cells obtained from the dishes which received both pPyMT1 and pPyST1 grew significantly more rapidly than those from the dishes which received pPyMT1 alone (data not shown). The results suggested that small T antigen enhanced the rate of anchorage-independent growth of cells transformed by middle T antigen. To examine this phenomenon further, we decided to introduce pPyST1 into a clone of NIH 3T3 cells expressing only middle T antigen and examine the effect of pPyST1 on middle-T-expressing cells. Any effect seen should be due to small T antigen. We chose a clone of MT3 for subsequent studies, since this clone of NIH 3T3 cells contains a single copy of functional middle-T-antigen-coding region (see below). This clone was isolated from one of the dense foci which appeared on a monolayer of NIH 3T3 cells after transfection of pPyMT1.

Transfection of pGVST into MT3 cells. Monolayers of MT3 cells were transfected with pGVST, and 12 clones of G418-resistant cells were isolated. They were designated MT3-(pGVST)cl-1 to cl-12. As a control, five clones of G418-resistant cells were also isolated from the culture of MT3 transfected with a backbone plasmid, pGV16, and were designated MT3(pGV16)cl-1 to cl-5. The presence of T antigens in these cells was examined by immunoprecipitation (Fig. 2). All the clones, except for MT3(pGVST)cl-12 (Fig. 2, lanes 17), expressed middle T antigen at about the same level as the MT3 cells did. Of the 12 clones which received pGVST, MT3(pGVST)cl-1, cl-2, cl-4, cl-5, cl-6, and cl-10 also expressed small T antigen at various levels (Fig. 2, lanes 6, 7, 9, 10, 11, and 15, respectively). A faint band in lanes 12 and 13 in Fig. 2, present at about the same position as small T antigen, was found to be a background protein in repeated experiments.

All these clones of cells were tested for anchorage-independent growth. They all grew in soft agar, including MT3(pGVST)cl-12, which showed very low levels of middle-T-antigen expression, although the growth of these cells was the slowest of all the clones tested. Nine days after plating in soft agar, colonies large enough to be visible by the naked eye began to show up in some of the plates (Fig. 3). By this time, a significantly larger proportion of cells had given rise to visible colonies in the plates which contained cells expressing both middle and small T antigens (Fig. 3C) than those in the plates which contained cells isolated after pGV16 transfection (Fig. 3A) or those isolated after pGVST transfection but not expressing small T antigen (Fig. 3B). There was no obvious correlation between the amount of small T antigen expressed and the rate of cell growth in soft agar. MT3(pGVST)cl-6 in Fig. 3C, which expresses small T antigen at a higher level than does MT3(pGVST)cl-4 or cl-5, did not have colonies as large as those by other clones expressing both middle and small T antigens in repeated

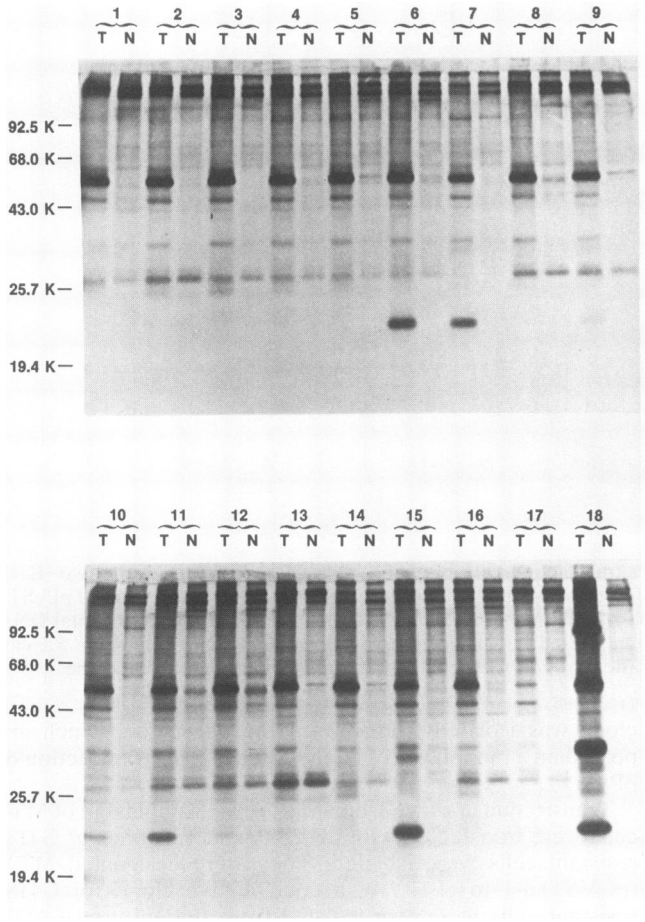

FIG. 2. T antigens immunoprecipitated from subclones of G418-resistant MT3 cells after pGVST transfection. Subconfluent cells in a 60-mm dish were labeled with 300 μCi of [^{35}S]methionine for 4 h. T antigens were immunoprecipitated from extracts of these cells with rat antitumor serum (T) or control nonimmune rat serum (N). Lanes 1 to 5, MT3(pGV16)cl-1 to cl-5; lanes 6 to 17, MT3(pGVST)cl-1 to cl-12; lane 18, a lane of rat cells transformed by polyomavirus T1A1 (21) as a size reference for the authentic large, middle, and small T antigens. Several molecular weight marker proteins were run in parallel lanes, and the positions are indicated on the left of each panel.

experiments. Since the growth-stimulating effect of small T antigen in MT3 cells was fairly reliably reproduced in several independent sets of experiments (see below), we suspected that something unusual might have happened to this clone. Subsequent studies revealed that one copy of pGVST was integrated in these cells and that a virus-cell junction occurred at a position very close to the region of the polyomavirus genome encoding the carboxy terminus of small T antigen (data not shown). Therefore, some sequence alterations might have occurred within the coding region of small T antigen in this clone. Further studies are required to clarify this point.

Since growth enhancement of MT3 cells in soft agar by small T antigen was not clearly demonstrated in one of six clones tested above, essentially the same experiment was performed with pPyST1 instead of pGVST. We were able to introduce a small-T-antigen gene and selected cells expressing the small T antigen by neomycin selection when MT3 cells were cotransfected with pPyST1 and pSV2*neo*, whereas we were unable to isolate any G418-resistant cells expressing small T antigen when NIH 3T3 cells were transfected with pPyST1 and pSV2*neo* (data not shown). Of 15 G418-resistant cell clones [MT3(pPyST)cl-1 to cl-15], 14 expressed small T antigen at various levels, as judged by immunoprecipitation (data not shown). All 14 clones, except for MT3(pPyST)cl-6, expressed about the same amount of middle T antigen as MT3 cells did. The MT3(pPyST)cl-6 cells expressed approximately one-half to one-third as much middle T antigen as the parental MT3 cells did. These clones of cells were suspended in soft agar. In all cases, except for MT3(pPyST)cl-6, small-T-antigen-containing cells grew much faster than MT3 cells did. MT3(pPyST)cl-6 cells formed about the same size of colonies as MT3 cells did, presumably because this subclone of MT3 cells expresses a smaller amount of middle T antigen and has a lower level of middle-T-antigen-associated protein kinase activity than the parental cells do. We observed large colonies formed by cells expressing both middle and small T antigens 10 days after plating (Fig. 4). We conclude firmly that small T antigen cooperates with middle T antigen in anchorage-independent growth of NIH 3T3 cells.

Influence of introduction of small-T gene on the structure and expression of middle-T gene in MT3 cells. Since pGVST or pPyST1 shares sequence homology with the middle-T-antigen gene present in MT3 cells, it was necessary to examine whether the small-T-antigen gene introduced into MT3 cells would interact with the middle-T-antigen gene and thereby alter the expression of middle T antigen. First, we compared the integrated middle-T-antigen gene in MT3 cells, before and after the introduction of the small-T-antigen gene, by Southern blot analysis. High-molecular-weight DNA prepared from each of the six clones expressing both middle and small T antigens shown in Fig. 2, as well as from MT3 cells, was digested with several restriction enzymes and probed for the polyomavirus specific sequence. The restriction patterns of MT3 and MT3(pGVST)cl-2 DNAs are shown as an example (Fig. 5). MT3 DNA contains two copies of polyomavirus-specific sequence, one hybridizing strongly (indicated as I in Fig. 5A, lane 1a) and one hybridizing weakly with the probe used (indicated as II in Fig. 5A, lane 1a). From the results shown in Fig. 5 and other restriction enzyme patterns, the most likely structures of these two polyomavirus sequences in MT3 DNA were deduced and are shown, indicating that MT3 contained a single copy of the functional middle-T-antigen-coding sequence (Fig. 5B).

It is clear that MT3(pGVST)cl-2 DNA contains the middle-T-antigen coding sequence exactly as found in parental MT3 cells (Fig. 5A, lanes 1b, 2b, 3b, and 4b). In addition, this DNA contains several copies of viral DNA, presumably representing the small-T gene. The 2.2-kilobase-pair fragment generated by double digestion with *Bam*HI and *Eco*RI and shown in Fig. 5A, lane 2b, probably corresponds to the internal fragment from *Bam*HI at nt 4632 to *Eco*RI at nt 1560 in the polyomavirus genome, containing the entire small-T-antigen-coding region as well as the viral promoter and enhancer elements (41).

MT3(pGVST)cl-1 and cl-6 contained a single copy of the small-T-antigen gene, while MT3(pGVST)cl-4, cl-5, and cl-10 contained about five, two, and eight copies, respectively, of small-T-antigen genes. In all cases, the middle-T-antigen gene present in MT3 cells and shown in Fig. 5 was present without any obvious alterations (data not shown).

To compare the levels of mRNA which encode middle T antigen before and after the introduction of the small-T gene, total cytoplasmic RNA was isolated from MT3,

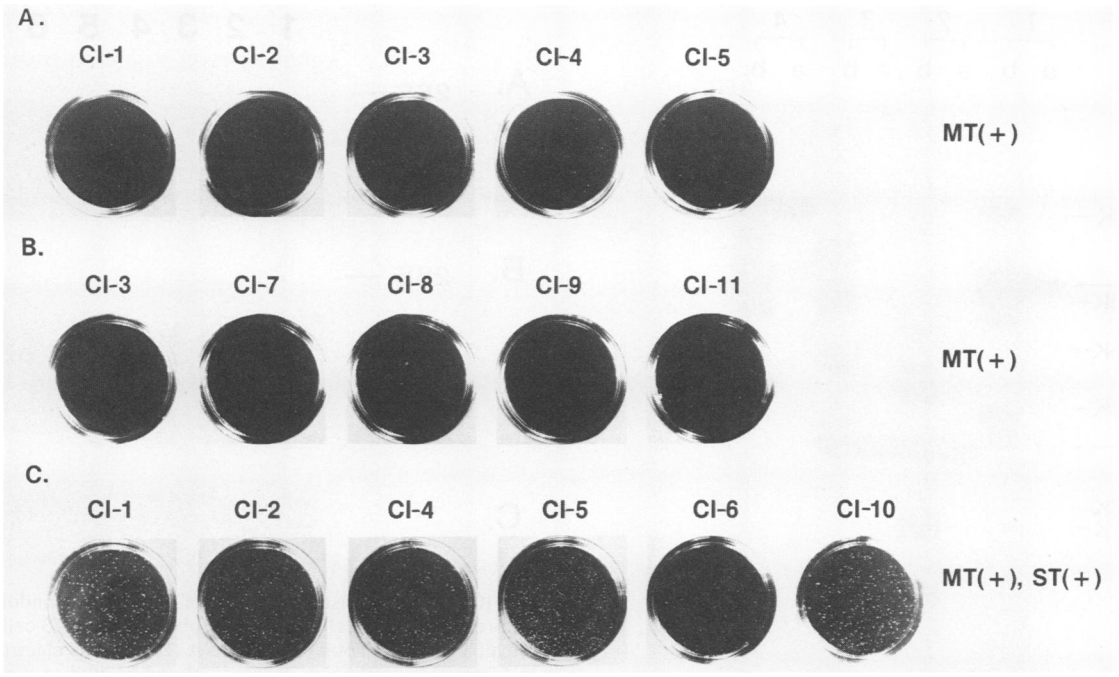

FIG. 3. Difference in number of visible colonies formed in soft agar by MT3 cells or subclones of MT3 cells expressing small T antigen. Cells (10^3) were suspended in semisolid medium (Dulbecco modified Eagle medium containing 10% calf serum and 0.33% agar [Difco]) and incubated in 60-mm dishes for 9 days. Dishes were fed with fresh medium every 2 to 3 days. (A) MT3(pGV16)cl-1 to cl-5; (B) MT3(pGVST)cl-3, cl-7, cl-8, cl-9, and cl-11 (small-T-antigen-negative clones); (C) MT3(pGVST)cl-1, cl-2, cl-4, cl-5, cl-6, and cl-10 (small-T-antigen-positive clones). MT(+), Middle T antigen positive; ST(+), small T antigen positive.

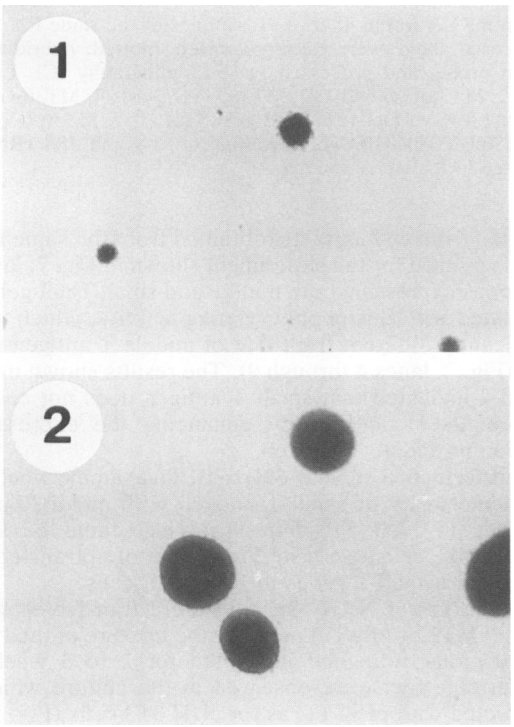

FIG. 4. Colony formation. Cells (10^3) were suspended in semisolid medium and incubated for 10 days. Colonies formed by MT3 cells (panel 1) and by MT3(pPyST)cl-8 cells (panel 2) were photographed with an inverted microscope with low-power magnification (×100).

MT3(pGV16)cl-1, and the six clones that expressed the middle and small T antigens mentioned above and was examined by Northern blot analysis. When we used a DNA fragment from *Bgl*I (nt 87) to *Pst*I (nt 484) of the polyomavirus genome as a radioactive probe, two mRNA species, 2.9 and 1.3 kilobases (kb) in size, were detected (data not shown). The 2.9-kb species is a predicted mRNA species for middle T antigen (25). When a DNA fragment from *Hin*dIII (nt 1656) to *Hin*cII (nt 2962) was used as a probe, only a 2.9-kb species was detected. Therefore, a 1.3-kb species is likely to be the one which terminated near the *Eco*RI site at nt 1550, where a cryptic polyadenylation site is present (13). Since the former probe could not distinguish between the mRNAs for middle and small T antigens, while the latter could detect only mRNA for middle T antigen, the latter probe was used for the comparison between the clones shown in Fig 6. We assume that the ratio of middle-T-antigen-specific 2.9-kb and 1.3-kb RNAs is constant in all cases. Figure 6 shows the 2.9-kb species present in RNA isolated from all the clones mentioned above. There is no significant difference in the amounts of the 2.9-kb mRNA between MT3 and the other clones (Fig. 6A). The levels of β-actin mRNA in the same RNA preparation were used as an internal control (Fig. 6B and C). Small variations in the levels of the expression among the clones were within experimental error and did not correlate with the presence or the absence of the small-T gene in the cells. We conclude that the introduction of the small-T gene does not significantly alter the level of mRNA expression for middle T antigen.

Since the tyrosine-specific protein kinase activity associated with middle T antigen is the only known biochemical function of middle T antigen and correlates well with the ability of the cells transformed by polyomavirus to grow in

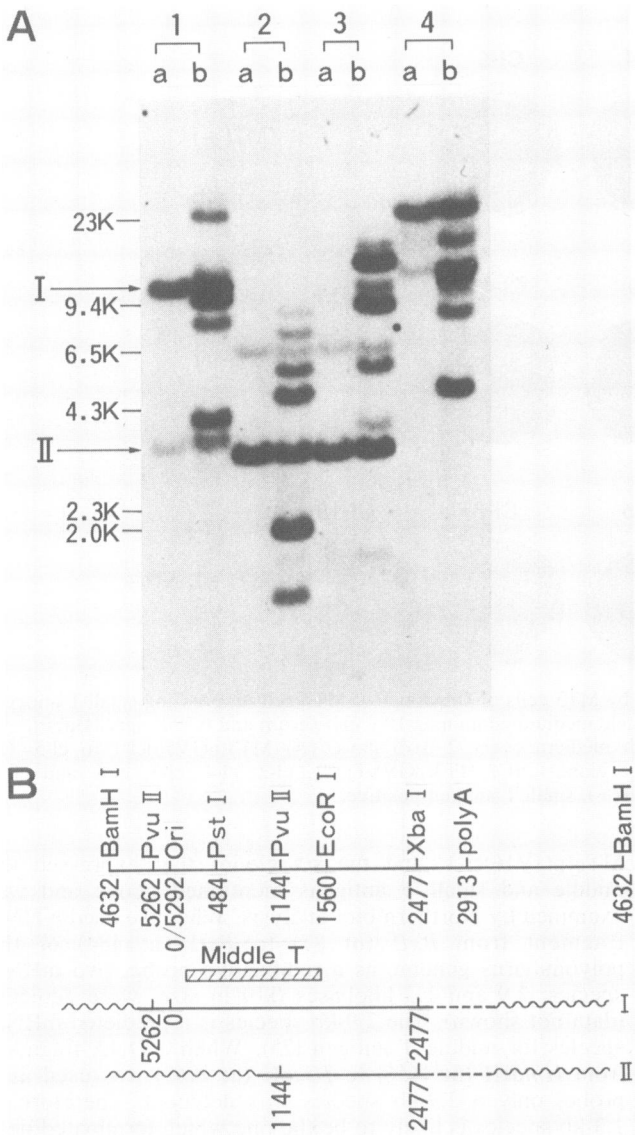

FIG. 5. (A) Comparison of integrated viral sequences in MT3 (a) and subclone MT3(pGVST)cl-2 (b) by Southern blot analysis. High-molecular-weight cellular DNA (20 μg) was restricted by *Bam*HI (lane 4), *Eco*RI (lane 3), *Xba*I (lane 2), or *Bam*HI and *Eco*RI (lane 1), and the fragments generated were separated in 0.6% agarose gel. After blotting, filters were hybridized with a nick-translated polyomavirus DNA fragment encompassing the coding regions for middle and small T antigens (from nt 4632 through nt 1550; see reference 41 for nucleotide numbering). *Hin*dIII-restricted fragments of lambda bacteriophage DNA were run in parallel as size markers, and the positions are indicated on the left. (B) Polyomavirus sequences in MT3 DNA deduced from restriction enzyme patterns. ——, Viral sequences; ∿∿∿, cellular sequences; -----, regions within which junctions between viral and cellular sequences lie.

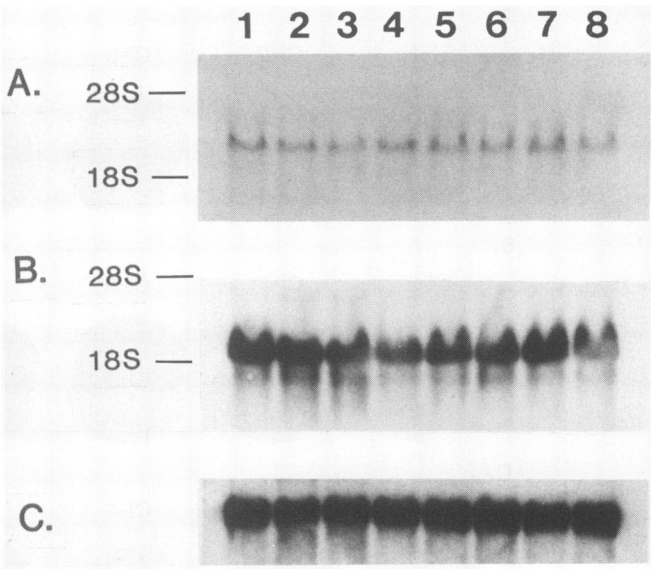

FIG. 6. Comparison of levels of mRNA for middle T antigen expressed in MT3 cells and in the subclones of MT3 cells expressing small T antigen. Total cellular RNA (20 μg) was electrophoresed in a formamide-containing agarose gel and blotted to a nitrocellulose filter. A nick-translated polyomavirus DNA fragment (from *Hin*dIII at nt 1656 to *Hin*cII at nt 2962) was hybridized to the filter and processed by autoradiography (A). After autoradiography, the filter was washed in 0.1× SSC containing 0.1% SDS at 90°C for 10 min to remove radioactive material, rehybridized with a nick-translated DNA fragment obtained from plasmid pA-1 which contained murine β-actin cDNA, and processed by autoradiography (B). To avoid distortion of the bands representing β-actin mRNA and unevenness of hybridization to that mRNA due to an excessive amount of the β-actin mRNA in the filter, 2-μg samples of the same RNA preparation used above were electrophoresed, blotted, hybridized with β-actin probe, and processed by autoradiography (C). Lanes: 1, MT3; 2, MT3(pGV16)cl-1; 3, MT3(pGVST)cl-1; 4, MT3(pGVST)cl-2; 5, MT3(pGVST)cl-4; 6, MT3(pGVST)cl-5; 7, MT3(pGVST)cl-6; 8, MT3(pGVST)cl-10. The positions of 28S and 18S rRNAs are indicated to the left of the gels.

soft agar, this enzyme activity was compared before and after the introduction of pGVST into MT3 cells.

Figure 7, lane 3 shows the level of phosphorylation of middle T antigen immunoprecipitated from a cell extract from a given number of MT3 cells. When twice the amount of cell extract was used, approximately twice the level of middle T antigen phosphorylation was observed (Fig. 7, lane 2). Under these conditions, middle T antigens immunoprecipitated from cell extracts (obtained from the same number of cells as used for the experiment shown in Fig. 7, lane 3) of six clones expressing both middle and small T antigens were associated with this phophorylating activity, which was not significantly different from that of middle T antigen in MT3 cells (Fig. 7, lanes 4 through 9). The results shown in Fig. 5, 6, and 7 indicated that small T antigen does not cooperate with middle T antigen by enhancing the expression or function of middle T antigen.

Transformation of NRK-52E cells. To examine whether the complementarity of small T antigen with middle T antigen observed in NIH 3T3 fibroblast cells could be seen in epitheliallike cells, we tested the effect of cotransfection of middle- and small-T genes in NRK-52E cells.

Monolayers of NRK-52E cells were transfected with either pPyMT1 or pPyST1 alone or the mixture of the two at a 1-to-10 molar ratio and incubated for 2 to 3 weeks. No recognizable foci were observed in the culture which was transfected with pPyST1, as for NIH 3T3 cells (Fig. 1D). In the culture transfected with pPyMT1, small, slow-growing foci appeared, whereas in the culture transfected with the mixture of the two, much faster growing dense foci appeared. In the culture which received pPyMT1, foci appeared 3 weeks after transfection (Fig. 8A). A higher mag-

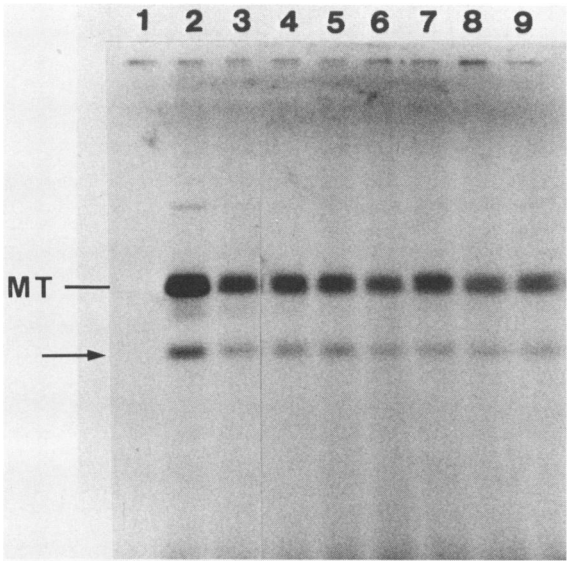

FIG. 7. Tyrosine protein kinase activities associated with middle T antigen of MT3 and its subclones expressing small T antigen. Cell extracts made from 5×10^5 cells (lanes 1 and 3 through 9) or 1×10^6 cells (lane 2) were subjected to immunoprecipitation with 10 μl of hamster anti-T serum (lanes 2 through 9) or nonimmune control hamster serum (lane 1) and the protein kinase assay described in Materials and Methods. Phosphorylated middle T antigen appeared as a doublet (37). The bands which appear below middle T antigen, indicated by an arrow, are most likely degradation products of middle T antigen. Lanes: 1, 2, and 3, MT3; 4, MT3(pGVST)cl-1; 5, MT3(pGVST)cl-2; 6, MT3(pGVST)cl-4; 7, MT3(pGVST)cl-5; 8, MTE(pGVST)cl-6; 9, MT3(pGVST)cl-10.

nification of the same focus is shown in Fig. 8B. The larger focus shown in Fig. 8C is typical of the foci which appeared in the culture transfected with the mixture of pPyMT1 and pPyST1 2 weeks after transfection (1 week before the focus in Fig. 8A was photographed). A higher magnification of the same focus is shown in Fig. 8D. Cells picked from foci shown in Fig. 8 expressed the appropriate T-antigen species (data not shown). A quite significant difference in the growth rate of the cells transformed by middle T antigen alone and by both middle and small T antigens observed here is in distinct contrast from the results obtained with NIH 3T3 cells (Fig. 1).

The anchorage-independent growth of these cells was then compared. Since NRK-52E cells did not survive when suspended in the soft agar used for NIH 3T3 cells, soft agarose was used instead for these cells. Cells obtained from several small foci and expressing middle T antigen did not divide in soft agarose in 4 weeks, while cells obtained from much larger foci and expressing both middle and small T antigens did grow moderately well under the conditions used. Typical examples with NRK-52E(MT)f-1 isolated from the former cultures and NRK-52E(MTST)f-1, f-2, and f-3 isolated from the latter cultures are shown in Fig. 9 and 10. NRK-52E(MT)f-1 cells expressed middle T antigen (Fig. 9A, lanes 1) which had associated tyrosine protein kinase activity (Fig. 9B, lanes 1). This clone of cells did not divide in soft agarose (Fig. 10A). NRK-52E(MTST)f-1, f-2, and f-3 all expressed middle and small T antigens (Fig. 9A, lanes 2, 3, and 4, respectively). The amount of middle T antigen expressed in these cells, especially in NRK-52E(MTST)f-1 and f-2 cells (Fig. 9A, lanes 2 and 3), was signficantly lower than that of NRK-52E(MT)f-1 (Fig. 9A, lanes 1). Middle-T-antigen-phosphorylating activities associated with these cells roughly paralleled the amounts of middle T antigen itself (Fig. 9B). NRK-52E(MT)f-1 and NRK-52E(MTST)f-1, f-2, and f-3 were cells picked from foci without further cell cloning. It is possible, therefore, that these lines may contain some untransformed cells. However, by the way we picked these cells, we were certain that contaminating cells, if any, could not be more than 10% of the total cell population. Therefore, it is quite unlikely that the conclusion obtained from the results shown in Fig. 9 could be affected by the fact that these cells did not go through rigorous cell cloning. All NRK-52E(MTST) cell clones tested here grew in soft agarose (Fig. 10, panels 2, 3, and 4). Thus the expression of middle T antigen alone in NRK-52E cells appears to be insufficient to induce anchorage-independent growth, even when middle-T-associated kinase activity was manifested at reasonable levels and simultaneous expression of middle and small T antigens seems to be required for it.

DISCUSSION

We have investigated whether structurally related middle and small T antigens of polyomavirus would cooperate in the in vitro transformation of established fibroblast and epitheliallike cell lines.

We demonstrated that the rate of anchorage-independent growth of NIH 3T3 fibroblast cells transformed by middle T antigen was enhanced dramatically when the small-T-antigen gene was introduced into the cells, although the effect of small T antigen on foci formation by middle T antigen on monolayers was not apparent. This enhancement of cell growth in semisolid medium was not due to increased expression of middle T antigen or enhanced tyrosine protein kinase activity associated with middle T antigen. NRK-52E epitheliallike cells expressing both middle and small T antigens formed quite significantly larger foci than those expressing only middle T antigen in a monolayer culture. Within the limits of our experiment, middle T antigen did not induce anchorage-independent growth when middle-T-antigen expression in NRK-52E cells and its associated tyrosine protein kinase activity were clearly detected. Middle and small T antigens together did induce cell growth in semisolid medium, even when the levels of the expression of middle T antigen and its associated tyrosine protein kinase activity were considerably lower than those in middle-T-antigen-expressing cells.

Since the small T antigen has a growth factor-like activity on NIH 3T3 cells (30), it is easy to understand that small T antigen stimulates the growth of middle-T-antigen-transformed NIH 3T3 cells, provided that the function of small T antigen is not expressed by middle T antigen. Does small T antigen express a subset of the functions of middle T antigen? The middle-T-antigen gene has been placed under the control of an inducible promoter. When the expression of middle T antigen was gradually increased by adding increasing amounts of the inducer, the size of the colonies formed by the cells gradually increased up to a certain point (31). Therefore, the size of the colonies is clearly influenced by the amount of middle T antigen. In our routine experiments, the average size of colonies formed by cells transfected with various amounts of the middle-T-antigen gene did not noticeably change, although the number of colonies was dependent on the amount of the DNA used. When we transfected cells with a mixture of middle- and small-T-antigen genes, a significant proportion of colonies grew to

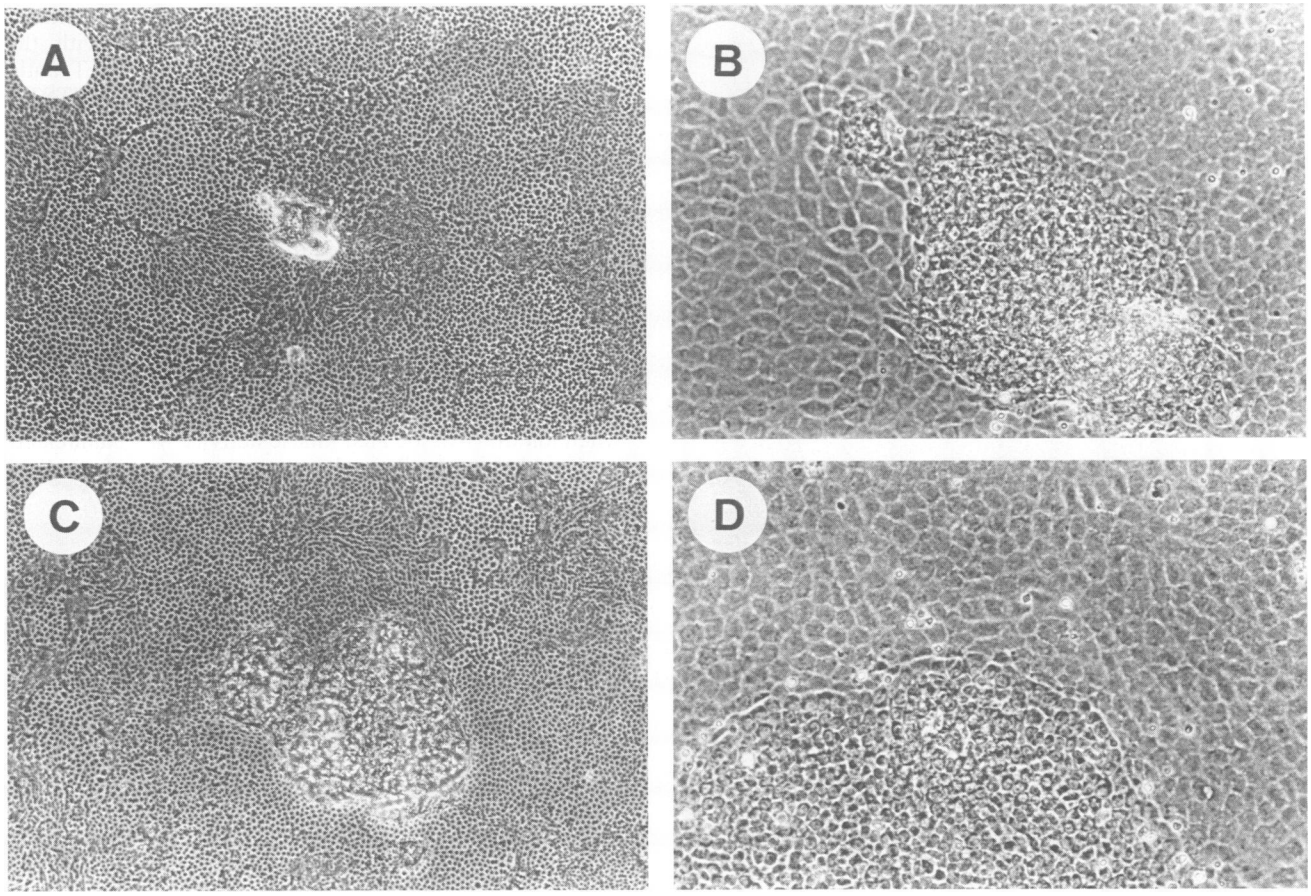

FIG. 8. Dense focus formed in monolayers of NRK-52E cells after transfection with pPyMT1 or the mixture of pPyMT1 and pPyST1. NRK-52E cells (10^5) were plated per 100-mm dish; 16 h after plating, they were transfected with 1 μg of pPyMT1 (A and B) or 1 μg of pPyMT1 and 10 μg of pPyST1 (C and D). Typical foci which appeared 2 weeks (C and D) or 3 weeks (A and B) later were photographed with a phase-contrast microscope. Magnification, ×100 for panels A and C and ×500 for panels B and D.

much larger sizes than those in the parallel culture, in which the cells were transfected only with the middle-T-antigen gene. As described in Results, randomly isolated cells from foci from the culture which received both middle- and small-T-antigen genes grew more rapidly than those picked from the culture which received only the middle-T-antigen gene. These observations prompted us to study the effect of the small-T-antigen gene on cells transformed by middle T antigen. The results clearly showed that the two genes cooperated in anchorage-independent growth of the cells in semisolid medium, suggesting that the function of small T antigen is not expressed by middle T antigen. The results from transformation experiments with NRK-52E cells further demonstrated that the function of small T antigen could not be replaced by the larger amount of middle T antigen (Fig. 9 and 10); hence, these two T antigens were indeed distinct. In addition, small T antigen did not increase the amounts of middle T antigen or enhance the levels of middle-T-antigen function. Therefore, the mechanisms by which these two proteins alter the growth properties of the cells must be entirely different.

It may not be surprising to find that the two T antigens do not share function, since intracellular localization of these two T antigens is quite different, middle T antigen being associated with the cellular membrane (21) and small T antigen being primarily in the nuclei (30). Middle T antigen has a membrane anchorage domain near its carboxy-terminal end, and removal of that domain abolishes the transforming ability of middle T antigen (6). It is still unknown whether middle T antigen devoid of that domain will localize to the nuclei and have a small-T-antigen-like function. It is also an open question whether the *hr*-t mutation, which is known to abolish the function of middle T antigen, would also eliminate the function of small T antigen. In any case, since the structure and the function of large T antigen are quite different from those of middle and small T antigen, our results imply that the early region of polyomavirus encoding three species of T antigens indeed has three distinct gene functions.

The complementarity between small and middle T antigen was more dramatically observed in NRK-52E than in NIH 3T3 cells. Middle T antigen alone did not induce anchorage-independent growth in NRK-52E cells. The notion that middle T antigen alone is insufficient to induce the full transformation phenotype was first suggested by Rassoulzadegan and others using primary rat embryo fibroblasts (32). It has been generalized that the transformation of established cell lines does not require gene functions other than that of middle T antigen, while the transformation of primary culture cells requires large- and probably small-T-antigen functions as well (10). Although the distinction between primary cells versus established cell lines seems to

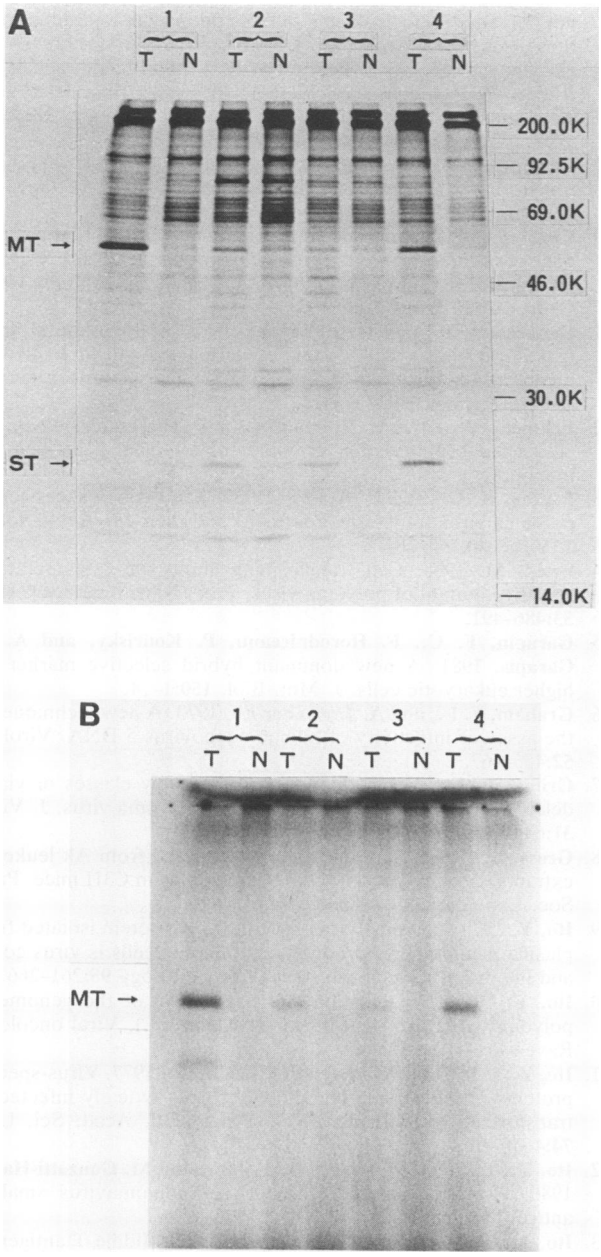

FIG. 9. Levels of expression of middle T antigen and its associated kinase activities in NRK-52E cells expressing only middle T antigen or middle and small T antigens. Four clones of NRK-52E cells were labeled with [^{35}S]methionine for 4 h. Parallel cultures were incubated similarly without radioactive labeling. [^{35}S]methionine-labeled T antigens were immunoprecipitated with rat antitumor serum and analyzed by SDS-PAGE and fluorography (A). Unlabeled middle T antigen was immunoprecipitated with hamster antitumor serum and was subjected to an in vitro protein kinase assay (B). Lanes: 1, NRK-52E(MT)f-1; 2, NRK-52E(MTST)f-1; 3, NRK-52E(MTST)f-2; 4, NRK-52E(MTST)f-3; T, anti-T serum treated; N, nonimmune control serum treated. MT, middle T antigen; ST, small T antigen. Protein size markers were run in a parallel lane, and the positions are indicated on the right.

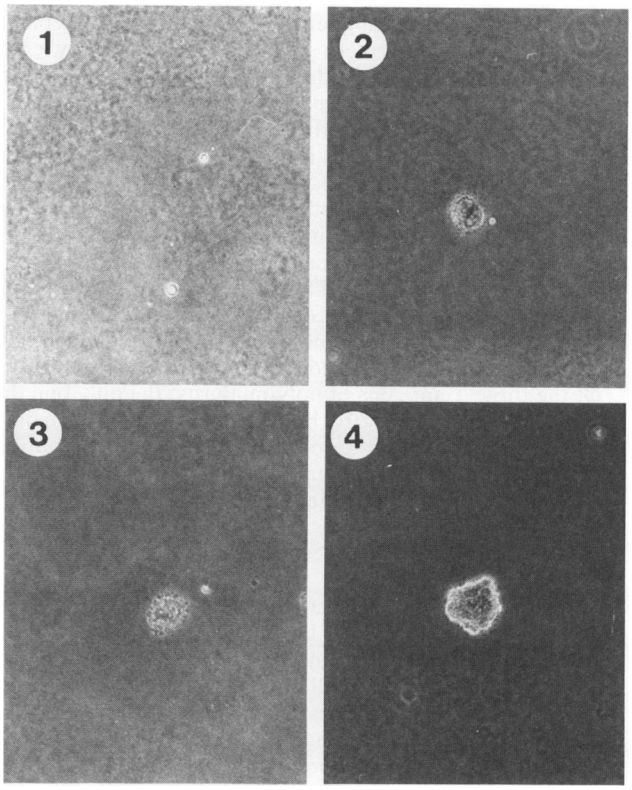

FIG. 10. Colonies formed by NRK-52E cells expressing middle and small T antigens in soft agarose. Cells (10^3) were suspended in soft agarose (Dulbecco modified Eagle medium containing 10% fetal calf serum and 0.3% SeaKem agarose (FMC Corp., Marine Colloids Div., Rockland, Maine]) and incubated for 2 weeks. Colonies formed were photographed with an inverted phase-contrast microscope with 150× magnification. Panels: 1, NRK-52E(MT)f-1; 2, NRK-52E(MTST)f-1; 3, NRK-52E(MTST)f-2; 4, NRK-52E(MTST) f-3.

be important, it must also be emphasized that most of the transformation studies have been performed with fibroblast cells. One possible reason for the inability of middle T antigen to induce anchorage-independent growth in NRK-52E cells is that the amounts of middle T antigen accumulated in these cells were at relatively low levels, so that the function of middle T antigen was not sufficiently expressed. Indeed, the levels of the expression of middle and small T antigens in NRK-52E cells were generally lower than those in NIH 3T3 cells. The amounts of $p60^{c\text{-}src}$ expressed and the pattern of tyrosine phosphorylation of cellular protein in NRK-52E cells would also have to be compared with those in NIH 3T3 cells. In considering the possible reason for inability of middle T antigen to transform NRK-52E cells, it would also be important to note that the number of epidermal growth factor receptors on NRK-52E cells is some 20-fold higher than that on NIH 3T3 cells (11). Epidermal growth factor has been shown to stimulate the activity of middle-T-antigen-associated tyrosine protein kinase activity when added to isolated plasma membranes or to intact cells externally (38). Although it is currently unknown how this activation occurs, it would be worth examining the correlation between the number of epidermal growth factor receptors on cells and the ability of middle T antigen to transform those cells in many different cell types. It is an entirely open question as to whether the limited transforming ability of middle T antigen on NRK-52E cells is generally observable in the transformation of certain types of epithelial cells.

While these possibilities need to be examined, it is striking to note that NRK-52E cells containing barely detectable

amounts of middle T antigen associated with only barely detectable levels of protein tyrosine kinase activity could grow in soft agarose when these cells were also expressing small T antigen. In this particular situation, middle-T-associated tyrosine protein kinase activity, which is the only known function of middle T antigen considered to be responsible for the induction of anchorage-independent growth in fibroblasts (31), is not sufficient to induce anchorage-independent growth of epitheliallike cells, and small T antigen appears to be at least as important as middle T antigen for that function. This situation is quite different from that with NIH 3T3 cells. There is no difficulty in isolating fully transformed mouse or rat fibroblast cell lines after transfection of the middle-T gene.

Of the three T antigens of polyomavirus, the only one that has been shown clearly to be responsible for the maintenance of transformed state of the cells is middle T antigen (23, 31). The effect of small T antigen on cell growth observed in the present studies seems to suggest that small T antigen also functions in such a manner as to be required continuously for the maintenance of a part of the transformed phenotype.

Each of the unique functions of the three T antigens (immortalization function of large T antigen, modification of $p60^{c\text{-}src}$ kinase activity by middle T antigen, and growth factor-like activity of small T antigen) is known to be intimately related to different aspects of oncogenic transformation. Polyomavirus, having these three functions together with the capability to transform epithelial cells, is, therefore, a unique tumor virus as a tool to study oncogenic cell transformation.

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