Isolation and Characterization of NIH 3T3 Cells Expressing Polyomavirus Small T Antigen

TETSUO NODA,[†] MASANOBU SATAKE,[†] TERRY ROBINS, AND YOSHIKI ITO^{†*}

Molecular Mechanisms of Carcinogenesis Laboratory, LBI-Basic Research Program, Frederick Cancer Research Facility, National Cancer Institute, Frederick, Maryland 21701

Received 10 February 1986/Accepted 20 June 1986

The polyomavirus small T-antigen gene, together with the polyomavirus promoter, was inserted into a retrovirus vector pGV16 which contains the Moloney sarcoma virus long terminal repeat and neomycin resistance gene driven by the simian virus 40 promoter. This expression vector, pGVST, was packaged into retrovirus particles by transfection of ψ 2 cells which harbor packaging-defective murine retrovirus genome. NIH 3T3 cells were infected by this replication-defective retrovirus containing pGVST. Of the 15 G418-resistant cell clones, 8 express small T antigen at various levels as revealed by immunoprecipitation. A cellular protein with an apparent molecular weight of about 32,000 coprecipitates with small T antigen. Immunofluorescent staining shows that small T antigen is mainly present in the nuclei. Morphologically, cells expressing small T antigen are indistinguishable from parental NIH 3T3 cells and have a microfilament pattern similar to that in parental NIH 3T3 cells. Cells expressing small T antigen form a flat monolayer but continue to grow beyond the saturation density observed for parental NIH 3T3 cells and eventually come off the culture plate as a result of overconfluency. There is some correlation between the level of expression of small T antigen and the growth rate of the cells. Small T-antigen-expressing cells form small colonies in soft agar. However, the proportion of cells which form these small colonies is rather small. A clone of these cells tested did not form tumors in nude mice within 3 months after inoculation of 10^6 cells per animal. Thus, present studies establish that the small T antigen of polyomavirus is a second nucleus-localized transforming gene product of the virus (the first one being large T antigen) and by itself has a function which is to stimulate the growth of NIH 3T3 cells beyond their saturation density in monolayer culture.

The early region of polyomavirus is essential for cell transformation and is transcribed into a single RNA species. Three alternative splicing events take place in this primary transcript, generating three mRNA species, each of which codes for a distinct transforming protein. They are called large, middle, and small T antigens (reviewed in reference 17). It has been suggested that each of these three transforming proteins contributes to cell transformation independently from the other two (2, 7). It is this feature which makes this virus unique in the study of oncogenic cell transformation. It is hoped, therefore, that the complex biological process of cell transformation could be dissected by analyzing the functions of these individual genes and their products.

Large T antigen is a DNA-binding protein (14) and is mainly localized to the nuclei of cells. In the lytic cycle, large T antigen is required for viral DNA replication and regulation of transcription. It is also known to *trans*-activate some cellular genes. In cell transformation, only the amino-terminal 40% of large T antigen is required (28). It has been suggested that large T antigen is able to convert primary rat embryo fibroblasts with limited doubling potential to established cell lines with unlimited doubling potential (28) without altering the morphology (22, 34). It is not clear which of the biochemical functions known to be associated with large T antigen is responsible for this immortalization or establishment function.

Middle T antigen is bound to the membrane and is primarily responsible for inducing the phenotype of transformed cells (16, 18, 21). Middle T antigen is associated with

an activity which phosphorylates middle T antigen on tyrosine in vitro (10, 32, 37). This activity is due to an associated cellular tyrosine protein kinase, a product of the proto-oncogene *c-src* (6). Middle T antigen has been shown to alter the protein tyrosine kinase activity of *c-src* upon binding (4). There is an intimate correlation between the level of the middle T antigen-associated protein tyrosine kinase activity and the extent of the transformed phenotype (37). Middle T antigen is necessary and sufficient to transform established rodent fibroblast cell lines (40), although it is not sufficient to transform primary rat embryo fibroblasts (27).

Small T antigen contains 196 amino acid residues. All except the carboxy-terminal 4 amino acids are included in the amino-terminal half of middle T antigen (Fig. 1). This raises the question of whether small T antigen has its own unique function which is not shared by middle T antigen. It has been reported that all three T-antigen genes, including the small T-antigen gene, are required for the transformation of primary rat embryo fibroblasts in vitro (7, 28). On the contrary, only two T-antigen genes, either large and middle T-antigen genes or middle and small T-antigen genes, appear to be required for in vivo tumor formation when inoculated into newborn rats (1, 2). Although somewhat contradictory, these results suggest some important role that small T antigen plays in cell transformation in vitro and in oncogenesis in vivo.

Very little is known about the biochemical function of small T antigen. One of the reasons for this lack of knowledge is that cells expressing only small T antigen have not been available until now, whereas cells expressing only large or middle T antigens have been widely available. It seems that the calcium phosphate coprecipitation method (42) results in the introduction of a large copy number of the

* Corresponding author.

[†] Present address: Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

FIG. 1. Overlapping coding region for three polyomavirus T antigens.

small T-antigen gene into cells. When small T antigen accumulates in large amounts in the cells, easy detachment of the cells from the plastic surface of the culture plates appears to occur (7, 45).

To isolate cells expressing only small T antigen, a retrovirus vector as helper-free, replication-defective virus containing the neomycin gene (13, 39) was used to transfer the small T-antigen gene into the recipient cells. This method was considered useful for introducing only a limited number of small T-antigen genes per cell, thereby limiting the production of small T protein. In addition, it was necessary to coselect small T-antigen-expressing cells by using a dominant selectable marker, since the phenotype of such cells was not known. Each gene was expressed by using an internal promoter within the retroviral genome (12). In this way, we were able to isolate NIH 3T3 cells expressing only small T antigen. In this paper, we describe some properties of such cells. The most important implication of this study is that small T antigen has its own biological function, namely growth-stimulating activity.

MATERIALS AND METHODS

Cells and medium. NIH 3T3 cells were obtained from Wallace Rowe, National Institutes of Health, Bethesda, Md. Clones MT-A and MT-B of NIH 3T3 cells expressing only middle T antigen were obtained by limiting dilution of the cells isolated as two independent dense foci after transfection of NIH 3T3 cells with the plasmid pPyMT1 (40) which induces the synthesis of only middle T antigen. To obtain clone MT/ST-A of NIH 3T3 cells which expresses both middle and small T antigens, G418-resistant cells were first selected after infection of NIH 3T3 cells with pGVST containing virus with helper Moloney murine leukemia virus (see below). Then the clone MT/ST-A was isolated by limiting dilution of cells obtained as dense focus after

transfecting the population of G418-resistant cells with pPyMT1. These cells were maintained in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories) containing 10% calf serum (Colorado Serum Co.).

Recombinant DNA technique. All the procedures used to construct pGVST were performed essentially as described previously (25). Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., New England BioLabs, Inc., and Boehringer Mannheim Biochemicals. T4 DNA ligase and calf intestine alkaline phosphatase were purchased from Boehringer Mannheim. All the enzymes were used as recommended by the manufacturers.

Helper-free defective retrovirus containing pGVST construct. On day 1, 3×10^5 ψ 2 cells (obtained from R. Mulligan, Massachusetts Institute of Technology, Cambridge, Mass.) which harbor packaging-defective murine leukemia virus genome (26) were plated per 100-mm plate and incubated in 10 ml of DMEM containing 10% calf serum. On day 2 (about 16 to 20 h after plating), 1 ml of 0.25 M CaCl_2 containing 2 μg of pGVST was added slowly to 1 ml of sterile $2 \times \text{HBS}$ (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 280 mM NaCl, 1.4 mM phosphate) (42) with constant agitation, poured into the culture plate without removal of the medium, and incubated for 16 h. On day 3, cells were washed once with DMEM containing 5% calf serum and fed with DMEM containing 10% calf serum. On day 4, G418 (13, 39) was added to the medium to 400 $\mu\text{g}/\text{ml}$. After this, the medium was changed every 3 days with DMEM containing 10% calf serum and 400 μg of G418 per ml. Approximately 60 to 100 G418-resistant colonies per plate became recognizable. These colonies were isolated on day 10. These G418-resistant colonies were pooled and seeded at 2×10^6 cells per T75 flask. After 24 h of incubation in the presence of 400 μg of G418 per ml, the medium was changed to DMEM containing 10% calf serum without G418. Cells were incubated further for 22 h. The culture superna-

FIG. 2. Structure of recombinant plasmids pGV16 (30) (a) and pGVST (b). The origin of the sequences is as follows: solid line together with long terminal repeat (LTR); Moloney murine sarcoma virus, open box with or without hatched area; SV40 or polyomavirus as indicated, solid box; pBR322, stippled box; neomycin resistance gene from transposon Tn5 (13, 39). The polyomavirus sequence from the *Bcl*I site (nucleotide 5021) to the *Bgl*I sites (nucleotide 87) containing the origin of replication was inserted at the *Bam*HI site in pGV16 after *Bam*HI linkers were attached at both ends of this fragment. The *Eco*RI site was present in the polylinker of pGV16 (30).

tant was collected and centrifuged for 4 min at $700 \times g$. This supernatant was used as virus stock. Virus stocks prepared this way usually contain about 10^4 infectious units per ml which can transfer G418 resistance to the recipient cells. To transfer pGVST to NIH 3T3 cells, we seeded the cells 10^5 cells per 100-mm plate in DMEM containing 10% calf serum. On day 2, cells were inoculated with 1 ml of virus preparation which contains $5 \mu\text{g}$ of polybrene per ml and were incubated for 2 h with occasional rocking. Infected cells were incubated in fresh DMEM containing 10% calf serum. On day 3, G418 was added to the medium to $400 \mu\text{g}/\text{ml}$ and the culture was further incubated. The medium was replaced with a fresh medium containing $400 \mu\text{g}$ of G418 per ml every day for 2 days and every 3 days thereafter. About 1,000 to 2,000 G418-resistant colonies per plate became recognizable on day 8. These G418-resistant cells were pooled, and 15 cell clones were isolated by limiting dilution.

Immunoprecipitation, PAGE, and fluorography. Immunoprecipitation of [^{35}S]methionine-labeled small T antigen, subsequent analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), and fluorography of the gels were performed as described previously (16). Briefly, subconfluent cells in a 60-mm plate (approximately 10^6 cells) were labeled with $300 \mu\text{Ci}$ of [^{35}S]methionine (800 Ci/mmol; New England Nuclear Corp.) in 2 ml of DMEM lacking methionine for 3 h at 37°C . Cell extracts made from these labeled cells were subjected to immunoprecipitation with rat anti-T-antigen serum obtained from tumor-bearing rats or nonimmune control rat serum (20). Immunoprecipitates were analyzed by sodium dodecyl sulfate-PAGE on 12% polyacrylamide gels.

Double-immunofluorescent staining of cells for small T antigen and microfilament. Cells grown on cover glass were fixed with 3.7% formaldehyde in phosphate-buffered saline for 20 min and permeabilized with 0.1% Nonidet P-40 in phosphate-buffered saline for 10 min. The fixed cells were reacted for 40 min at 37°C with rat monoclonal antibodies C1 or C4 (9), which recognize the amino-terminal common region of three T antigens (36). Culture supernatant of hybridoma cells was used undiluted. Ascites fluid was di-

luted 50-fold with phosphate-buffered saline. After extensive washing, cells were reacted for 40 min at 37°C with appropriately diluted fluorescein-tagged anti-rat immunoglobulin G rabbit immunoglobulin G (Cooper Biomedical, Inc.) and rhodamine-tagged phalloidin (43) (Molecular Probes, Inc.). After being washed with phosphate-buffered saline, the cells on cover glass were mounted on a slide glass with buffered-glycerol mounting medium and viewed through an epifluorescence microscope. Pictures were taken on Kodak Tri-X film with appropriate filters for fluorescein or rhodamine.

Chemicals. The drug G418 (13) was obtained from GIBCO Laboratories. Bacto-Agar (Difco Laboratories) was used for soft-agar assay of anchorage-independent cell growth. Polybrene (Sigma Chemical Co.) was used for better adsorption of virus to cells.

RESULTS

Construction of a retrovirus vector which expresses polyomavirus small T antigen. Figure 2 shows a backbone retrovirus vector, pGV16 (30), which contains an origin region of polyomavirus from *Bcl*I to *Bgl*I (for polyomavirus genetic map, see reference 38). This 358-base-pair *Bcl*I-*Bgl*I fragment has *Bam*HI linkers at both ends and is cloned at the *Bam*HI site present in the polylinker located at the junction between the sequence derived from Moloney murine sarcoma virus and the plasmid pBR322 (about 8 o'clock in Fig. 2a). Small T antigen gene is derived from plasmid pPyST1 (45), which contains the entire polyomavirus genome lacking the intron for small T-antigen gene cloned at the *Bam*HI site of pAT153. The 2.2-kilobase *Bam*HI-*Eco*RI fragment of pPyST1 containing the coding region for small T antigen and the early promoter was isolated. The 358-base-pair *Bcl*I-*Bgl*I fragment which contains *Bam*HI linker at both ends was removed from pGV16 by cleaving with *Bam*HI and *Eco*RI at positions A and the *Eco*RI site (Fig. 2) and was replaced with the 2.2-kilobase *Bam*HI-*Eco*RI fragment of pPyST1. The polylinker in pGV16 contains an *Eco*RI site which accepted one end of the 2.2-kilobase polyoma fragment. In this construct, each gene was expressed by using an internal

FIG. 3. Autoradiogram of immunoprecipitated [^{35}S]methionine-labeled small T antigen. Fifteen G418-resistant cell clones isolated after infection of NIH 3T3 cells with retrovirus containing pGVST were labeled with [^{35}S]methionine. Immunoprecipitation of small T antigen, sodium dodecyl sulfate-PAGE, and fluorography were performed as described previously (16). The numbers 1 to 15 indicate clones 1 to 15, respectively. The positions of molecular weight markers are indicated. Symbols: T, immunoprecipitated with anti-T antigen serum; N, immunoprecipitated with nonimmune control serum.

promoter within the retroviral genome as described previously for avian retrovirus vectors (12). This plasmid is named pGVST and is shown in Fig. 2b.

Isolation of NIH 3T3 cells expressing small T antigen. pGVST was introduced into NIH 3T3 cells as a helper-free defective virus, as described in Materials and Methods. Of 15 G418-resistant cell clones isolated, 8 expressed 21-kilodalton (kDa) protein at various levels as revealed by immunoprecipitation (Fig. 3). This 21-kDa protein comigrates with authentic small T antigen present in polyomavirus-transformed T1A1 rat cells (18). [^{35}S]methionine-

labeled 21-kDa protein produced in clone 11 cells (Fig. 3) was gel purified and analyzed by tryptic peptide fingerprinting. It was confirmed that the fingerprint of the 21-kDa protein is indistinguishable from that of genuine small T antigen (data not shown). NIH 3T3 cells which express only small t antigen were also isolated by using murine leukemia virus as a helper without using ψ 2 cells. COP-5 cells, which constitutively express polyomavirus large T antigen (41), were infected with Moloney murine sarcoma virus. The infected COP-5 cells were transfected with pGVST and incubated for 48 h. The culture supernatant of these cells was used as a virus stock. NIH 3T3 cells were infected with this virus, and G418-resistant cells were selected. The clones ST-A and ST-B of NIH 3T3 cells that are shown in Fig. 4 were obtained this way (see below). The details of this method for other genes have been described elsewhere (30).

In addition to small T antigen, there is at least one component, the 32-kDa protein, which is specifically immunoprecipitated with the anti-T serum. This 32-kDa protein is more clearly shown in Fig. 4, in which it is always observed in clones which express small T antigen (ST-A, ST-B; arrow in lanes 2 and 3) and is absent in those expressing middle T antigen only (MT-A, MT-B; arrow in lanes 5 and 6). The 32-kDa protein is also present in the clone in which both middle and small T antigens are present (MT/ST-A; lane 4). These results suggest that the 32-kDa protein is not recognized directly by the anti-T antigen serum used but is coprecipitated with small T antigen, probably because it is associated with small T antigen. Figures 3 and 4 also show protein bands at positions 43K and 35K which appear to be specific to anti-T serum. However, the 43-kDa protein is clearly not bound to any T antigen, since it is present in the lanes in which there is no T antigen. The 35-kDa protein appears to be in the same category. The antiserum used must contain antibody activity against these cellular components.

Subcellular localization of small T antigen. Clone 11 of NIH 3T3 cells expressing small T antigen (Fig. 3) was found to have the highest level of small T antigen among the clones that we isolated. Using this clone 11, subcellular localization of small T antigen was examined by immunofluorescence.

FIG. 4. Autoradiogram of [^{35}S]methionine-labeled cellular protein coprecipitated with small T antigen. [^{35}S]methionine-labeled cell extracts were subjected to immunoprecipitation for polyomavirus T antigen, sodium dodecyl sulfate-PAGE and fluorography were done as described previously (16). Lanes: 1, NIH 3T3 cells; 2, clone ST-A of NIH 3T3 cells expressing small T antigen; 3, clone ST-B of NIH 3T3 cells expressing small T antigen; 4, clone MT/ST-A of NIH 3T3 cells expressing both middle and small T antigens; 5, clone MT-A of NIH 3T3 cells expressing only middle T antigen; 6, clone MT-B of NIH 3T3 cells expressing only middle T antigen. The arrow on the left indicates the 32-kDa cellular protein.

FIG. 5. Immunofluorescent labeling of small T antigen and phalloidin labeling of microfilament of clone 11 of NIH 3T3 cells expressing small T antigen. (A) Fluorescein labeling of the cells with anti-T antigen rat monoclonal antibody C-1 (9) and anti-rat immunoglobulin G rabbit immunoglobulin G. (B) Cells in panel A labeled with rhodamine-phalloidin. (C) NIH 3T3 cells labeled with rhodamine-phalloidin.

Monoclonal antibodies which recognize the amino-terminal common region of three species of T antigens (9) detect small T antigen in the nuclei (Fig. 5A). The staining pattern, i.e., diffuse fluorescence in the nucleoplasm and lack of fluorescence in the nucleoli, is very similar to that of large T antigen. The following additional experiments confirmed this nuclear fluorescence. A plasmid DNA, pPyST1, was microinjected into the nuclei of NIH 3T3 cells. The immunofluorescent staining pattern of T antigen in the cells at 48 h after microinjection was indistinguishable from that shown in Fig. 5A (data not shown). Although there was some fluorescence in the cytoplasm, similar fluorescence was also present in the parental NIH 3T3 cells (data not shown). It is therefore unclear whether some small T antigen is also present in places other than the nuclei. There are some variations in the intensity of nuclear fluorescence among members of the cell population. It is not clear at present what causes this variation. We tested many other small T-antigen-expressing cell clones by immunofluorescence. Essentially the same nuclear labeling pattern was observed in all cases tested.

Figure 5B shows bundle formation of actin-containing microfilament of the same set of cells shown in panel A. The cells expressing small T antigen have a well developed microfilament bundle pattern which is indistinguishable from that in parental NIH 3T3 cells (Fig. 5C). The results suggest that small T antigen has little, if any, effect on microfilament bundle formation in NIH 3T3 cells.

Growth characteristics of cells expressing small T antigen.

NIH 3T3 cells expressing small T antigen were morphologically indistinguishable from parental NIH 3T3 cells. When they approached confluency, cell-to-cell contact occurred normally and they formed a flat cell sheet as parental cells did. However, unlike parental cells, cells expressing small T antigen did not stop growing at confluency. They kept growing beyond the saturation density of normal NIH 3T3 cells and eventually came off the plates, while parental NIH 3T3 cells stopped growing at their saturation density and stayed as a monolayer for some weeks. Although the cell density increased, small T-antigen-expressing cells did not pile up on top of each other. Figure 6 shows the growth curve, in DMEM containing 10% calf serum, of each of the 15 clones shown in Fig. 3. Clones 1, 2, 5, 10, 12, 14, and 15, which did not express small T antigen, stopped growing after reaching their saturation densities and formed a flat

monolayer. Despite the fact that these clones underwent infection and neomycin selection, they all retained the growth characteristics of their parental cells. Some differences in saturation density observed among the clones might

FIG. 6. Growth curve of NIH 3T3 cells expressing polyomavirus small T antigen. A total of 2×10^5 cells of each clone, obtained by infection of retrovirus containing pGVST to NIH 3T3 cells, were plated in 60-mm dishes and incubated in DMEM-10% calf serum. The culture medium was changed every 3 days. Cells were trypsinized and counted on days 2, 4, and 6 by dye exclusion.

FIG. 7. Flat, dense foci formed by small T-antigen-expressing cells over the background of a flat NIH 3T3 cell sheet. Eighty cells of each clone mixed with 10^5 NIH 3T3 cells were plated in 60-mm dishes and incubated in DMEM-10% calf serum for 10 days. Cells were analyzed after Giemsa staining. Upper eight panels: NIH 3T3 cell clones not expressing small T antigen. The upper left corner shows parental NIH 3T3 cells. Lower eight panels: NIH 3T3 cell clones expressing small T antigen.

be due to clonal variations present in the parental cell population.

Clones 3, 4, 6, 7, 8, 9, 11, and 13 produced small T antigen. In all cases, cells grew beyond the saturation density of parental NIH 3T3 cells. The drop in cell number seen between days 4 and 6 was due to the detachment of cells as a result of overgrowth. There was some correlation between the amount of small T antigen produced in the cells and the growth rate of the cells. For example, clones 4, 7, and 11 produced low, intermediate, and high levels, respectively, of small T antigen (Fig. 3), and these three clones grew increasingly more rapidly (Fig. 6). Although the growth curve of clone 4 shown in Fig. 6 is similar to that of clones that do not express small T antigen, we were unable to maintain clone 4 as a cell sheet for long after the cells reached confluency.

This increased growth potential of small T-antigen-producing cells beyond saturation density can also be seen in Fig. 7, which shows dishes in which a small number of small T-antigen-expressing cells were mixed with a large excess of parental NIH 3T3 and incubated until the cells reached confluency. The upper half of Fig. 7 shows the clones of cells not expressing small T antigen. These cells, including parental NIH 3T3 cells, formed a flat monolayer with very few background foci. The lower eight panels represent cells expressing small T antigen. In these cases, clusters of small T-antigen-expressing cells can be seen as flat but dense foci over the background of a flat sheet of NIH 3T3 cells.

Since small T antigen was found to stimulate the growth of cells in monolayer, it is interesting to test whether small T antigen induces anchorage-independent cell growth. The upper half of Fig. 8 shows the clones of cells not expressing small T antigen and suspended in soft agar, while the lower half represents those expressing small T antigen and suspended in soft agar. The cells shown in the upper half of Fig. 8 did not undergo more than two to three cell divisions in the 4-week period. On the contrary, cells expressing small T antigen formed small colonies in soft agar. These colonies usually contained about 20 to 30 cells after the 4-week incubation. However, the properties of cells which formed small colonies was rather small, ranging from about 0.1 to 10%, depending on the clones. In this case, we did not see a clear correlation between the amount of small T antigen produced and the frequency or size of small colonies formed in soft agar. Eight of such small colonies were isolated from three different clones of small T-antigen-expressing cells. Only one of them formed small colonies at rather high frequency, namely about 30%, while others showed a low frequency of plating in soft agar, similar to that of the parental clones, suggesting that a low frequency of colony formation by cells expressing small T antigen is a stable property of such cell clones (data not shown).

Small T-antigen-expressing cells were then tested to see whether they formed tumors when inoculated into nude mice. When the clone expressing the largest amounts of small T antigen, namely clone 11, was used, no tumors were observed during the 3 months after the inoculation of 10^6 cells per nude mouse.

DISCUSSION

By introducing the small T-antigen gene into cells with a retrovirus vector as replication-defective retrovirus, we were able to isolate NIH 3T3 cells expressing only polyomavirus small T antigen. The reason we were able to isolate such cells, while previous attempts by others (7, 45) using the calcium phosphate coprecipitation method were unsuccessful, is most likely the introduction of the small T-antigen gene into cells in a helper-free retrovirus form which limits the number of gene copies introduced into cells in the present studies. Thus, the amount of small T antigen produced in the cells was below the toxic level. About half of the cell clones which expressed the neomycin gene also expressed the small T-antigen gene. It was not clear why the other half of the clones that expressed the neomycin gene did not express small T antigen. It is likely that a part or all of the small T-antigen gene is deleted in these cases. Precise analysis of the structure of the integrated pGVST is necessary to clarify this.

By examining the properties of these small T-antigen-expressing cells, we were able to study the subcellular localization of small T antigen and its effects on microfilament bundle formation, cell morphology, and growth characteristics.

Segawa and Ito (35) reported that small T antigen was recovered mainly in the cytoplasmic soluble fraction in cell fractionation studies. Immunofluorescent staining in the present studies revealed, however, that it is present mainly in the nuclei (Fig. 4). These apparently contradictory results suggest that small T antigen is present in the nuclei, not bound tightly to DNA or other nuclear components, but in a soluble form. The immunofluorescent staining pattern of small T antigen is indistinguishable from that of large T antigen. It is interesting to note that, although both large and

FIG. 8. Colony formation in soft agar of the clones of cells expressing and not expressing small T antigen. Cells (10^3) of 15 cell clones (Fig. 3) as well as parental NIH 3T3 cells were suspended in 0.33% agar in 60-mm plastic culture plates and incubated for 4 weeks at 37°C by the method of MacPherson and Montagnier (24). Cells cultured in suspension in soft agar were photographed through using a microscope with low-power magnification. Upper eight panels: NIH 3T3 cell clones not expressing small T antigen. The upper left corner shows parental NIH 3T3 cells. Lower eight panels: NIH 3T3 cell clones expressing small T antigen.

small T antigens show a similar immunofluorescent staining pattern, large T antigen, a DNA-binding protein, stays in the nuclei, while small T antigen easily comes out of the nuclei during the cell fractionation procedures. This may suggest that small t antigen is not a DNA-binding protein. In any event, it is important to note that polyomavirus transforming genes produce two distinct gene products, both of which are localized mainly to the nuclei. It has been shown that large T antigen has signal peptides necessary for its nuclear localization (29). The positions of such signals are outside the large T-small T common region. A similar peptide sequence does not appear to be present in the small T-antigen unique region. It remains to be seen whether small T antigen has a different type of signal for nuclear localization. Small T antigen of simian virus 40 (SV40), which has a high degree of amino acid sequence homology with polyomavirus small T antigen, has been shown to be present both in the nuclei and in the cytoplasm (3, 11).

Rodent fibroblast cells, after transformation by polyomavirus, usually lose well-developed actin-containing microfilament bundles and tend to show diffuse actin staining, particularly in membrane ruffles and at the edges of the cells. Established lines of rodent fibroblasts transformed by middle T antigen alone contain a greatly reduced amount of microfilament bundles indistinguishable from that seen in polyomavirus-transformed cells (19, 23). In contrast, the hr-t mutants, which induce normal large T antigen but not middle or small T antigens, do not alter the microfilament bundle pattern (19, 34). Using viral mutants which induce normal

large and small T antigens but not middle T antigen, Liang et al. (23) and Ito et al. (19) deduced that both large and small T antigens probably do not have any effect on microfilament organization. We have shown unambiguously in this paper that small T antigen has little, if any, effect on microfilament bundling. It is interesting to note that SV40 small t antigen has been reported to alter microfilament pattern (3, 15). Considering the high degree of sequence homology between the two small T antigens, it will be interesting to examine the significance of this difference.

Polyomavirus small T antigen has been transiently overexpressed in monkey CV-1 cells by Zhu et al. (45), who used SV40-polyoma recombinant virus. They observed that polyomavirus small t antigen is localized both in the nuclei and in the cytoplasm, causes drastic changes in morphology, and makes cells detach easily. It seems that the difference in the results is attributable to differences in the two experimental systems, monkey epithelial cells (CV-1) versus mouse fibroblasts and overexpression versus low-level expression.

Rundell and co-workers have reported in their series of papers that SV40, BK virus, human papovavirus, and polyomavirus small T antigens are bound to two cellular proteins of 32 and 56 kDa (5, 31, 44). We are able to show that small T antigen and the 32-kDa cellular protein are coprecipitated with anti-T antibodies, probably because they are bound. The 32-kDa protein that we observed might correspond to the 32-kDa protein described by Rundell and co-workers. It is not clear in Fig. 4 whether a 56-kDa protein

is coprecipitating with polyomavirus small T antigen because of the high background. It remains to be elucidated whether the association of small T antigen with either the 32-kDa or the 32- and 56-kDa cellular proteins is important for small T antigen to exert its function.

Small T antigen has little or no effect on the morphology of NIH 3T3 cells. We made the most important observation in the present studies in finding out the function of small T antigen when we compared the growth characteristics of cells expressing small T antigen with those of parental cells. NIH 3T3 cells expressing small T antigen form a flat monolayer at confluency. However, these cells are driven to grow without stopping at the saturation density of parental cells. Small T antigen is also able to induce anchorage-independent growth in NIH 3T3 cells, although this effect is weak. Consistent with this weak activity in inducing anchorage-independent growth, cells expressing small T antigen do not form tumors in nude mice. These properties of NIH 3T3 cells containing small T antigen are reminiscent of those of NIH 3T3 cells stimulated to grow by mitogenic growth factors, such as epidermal growth factor or platelet-derived growth factor (reviewed in reference 33). These growth factors are usually mitogenic to the cells in monolayer culture and increase the cell density. Except for some transforming growth factors such as TGF- α (8), however, they do not usually stimulate cells to divide when the cells are suspended in soft agar. Therefore, we suggest that small T antigen has a growth factor-like activity and enables cells to overcome a growth-inhibitory effect caused by high cell density. It remains to be seen whether small T antigen will induce cellular DNA synthesis in quiescent NIH 3T3 cells. It has been suggested that SV40 small T antigen also has a function associated with cellular growth control, as discussed by Bossert et al. (5).

It is unlikely that small T antigen stimulates cell growth by acting on cells externally, since small T antigen is present in the nuclei. Although we have not tested whether small T antigen is also released into the culture medium, preliminary results suggest that the culture medium of small T-antigen-expressing cells does not contain an activity which promotes the growth of NIH 3T3 cells, suggesting that the growth-stimulating activity of small T antigen that we observed is not due to action of small T antigen from outside the cells. Since small T antigen has a growth factor-like growth-promoting activity and is present in nuclei, it is tempting to speculate that small T antigen may be involved in a nuclear event that regulates cellular DNA synthesis. Since signals from activated growth-factor receptors induce cellular DNA synthesis and cell division, small T antigen might share with growth-factor receptors some nuclear mechanism by which cellular DNA synthesis is induced.

The fact that small T antigen has its own independent function raises the question of whether middle T antigen also has that function, since the entire polypeptide chain of small T antigen except for four carboxy-terminal amino acids is completely included in middle T antigen (Fig. 1). We have not yet done the experiments which directly address that question. However, we have observed that the addition of small T antigen to middle T-antigen-expressing cells results in a dramatic enhancement of the growth rate of the cells in soft agar without enhancing the activity associated with middle T antigen. The results suggest that middle T antigen does not exert small T-antigen function. This point, namely the question of whether small T antigen has its own unique function not shared by large or middle T antigen, is the subject of our paper in preparation.

We have established that small T antigen is the second polyomavirus transforming gene product which is localized to the nuclei. Since the behavior of the cells expressing only large T antigen observed previously (22, 34) and that of small T antigen observed in the present studies are very different and the primary structures of the unique region of large and small T antigens are very different (Fig. 1), we assume that the biochemical function of large and small T antigen will be very different, too. We will continue our effort to elucidate whether large and small T antigens contribute to the process of cell transformation by entirely different mechanisms.

ACKNOWLEDGMENTS

We thank R. Kamen for plasmids pPyST1 and pPyMT1 and COP-5 cells. R. Mulligan for ψ 2 cells, and B. E. Griffin for a set of rat monoclonal antibodies against polyomavirus T antigens.

The research was sponsored by the National Cancer Institute, under contract no. NO1-CO-23909 with Litton Bionetics, Inc.

LITERATURE CITED

- Asselin, C., C. Gelinas, and M. Bastin. 1983. Role of the three polyoma early proteins in tumorigenesis. *Mol. Cell. Biol.* 3:1451-1459.
- Asselin, C., C. Gelinas, P. E. Branton, and M. Bastin. 1984. Polyoma middle T antigen requires cooperation from another gene to express the malignant phenotype in vivo. *Mol. Cell. Biol.* 4:755-760.
- Bikel, I., T. M. Roberts, M. T. Bladon, R. Green, E. Amann, and D. M. Livingston. 1983. Purification of biologically active simian virus 40 small tumor antigen. *Proc. Natl. Acad. Sci. USA* 80:906-910.
- Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge. 1984. Enhancement of cellular *src* gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. *Cell* 38:767-777.
- Bossert, A., P. Mulgaonkar, and K. Rundell. 1985. Interaction of simian virus 40 small-T antigen produced in bacteria with 56K and 32K proteins of animal cells. *J. Virol.* 56:325-327.
- Courtneidge, S. A., and A. E. Smith. 1983. Polyoma virus transforming protein associates with the product of the *c-src* cellular gene. *Nature (London)* 303:435-439.
- Cuzin, F., M. Rassoulzadegan, and L. Lemieux. 1984. Multigenic control of tumorigenesis: three distinct oncogenes are required for transformation of rat embryo fibroblasts by polyoma virus, p. 109-116. In G. F. Vande Woude, A. J. Levine, W. C. Topp, and J. D. Watson (ed.), *Cancer cells*, vol. 2. Oncogenes and viral genes. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Delarco, J. E., and G. J. Todaro. 1978. Growth factors from murine sarcoma virus-transformed cells. *Proc. Natl. Acad. Sci. USA* 75:4001-4005.
- Dilworth, S. M., and B. E. Griffin. 1982. Monoclonal antibodies against polyoma virus tumor antigens. *Proc. Natl. Acad. Sci. USA* 79:1059-1063.
- Eckhart, W., M. A. Hutchinson, and T. Hunter. 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. *Cell* 18:925-933.
- Ellman, M., I. Bikel, J. Figge, T. Roberts, R. Schlossman, and D. M. Livingston. 1984. Localization of the simian virus 40 small T antigen in the nucleus and cytoplasm of monkey and mouse cells. *J. Virol.* 50:623-628.
- Emerman, M., and H. M. Temin. 1984. Genes with promoters in retrovirus vectors can be independently suppressed by an epigenetic mechanism. *Cell* 39:459-467.
- Garapin, F. C., F. Horodniceanu, P. Kourisky, and A. C. Garapin. 1981. A new dominant hybrid selective marker for higher eukaryotic cells. *J. Mol. Biol.* 150:1-14.
- Gaudray, P., C. Tyndall, R. Kamen, and F. Cuzin. 1981. The high affinity binding site on polyoma virus DNA for the viral large-T protein. *Nucleic Acids Res.* 9:5697-5710.

15. **Graessman, A., M. Graessman, R. Tjian, and W. C. Topp.** 1980. Simian virus 40 small-T protein is required for loss of actin cable networks in rat cells. *J. Virol.* **33**:1182–1191.
16. **Ito, Y.** 1979. Polyoma virus-specific 55K protein isolated from plasma membrane of productively infected cells is virus coded and important for cell transformation. *Virology* **98**:261–266.
17. **Ito, Y.** 1980. Organization and expression of the genome of polyoma virus, p. 447–480. *In* G. Klein (ed.), *Viral oncology*. Raven Press, New York.
18. **Ito, Y., J. R. Brocklehurst, and R. Dulbecco.** 1977. Virus-specific proteins in the plasma membrane of cells lytically infected or transformed by polyoma virus. *Proc. Natl. Acad. Sci. USA* **74**:4666–4670.
19. **Ito, Y., Y. Hamagishi, K. Segawa, T. Dalianis, E. Appella, and M. Willingham.** 1983. Antibodies against a nonapeptide of polyoma virus middle T antigen: cross-reaction with a cellular protein. *J. Virol.* **48**:709–720.
20. **Ito, Y., N. Spurr, and R. Dulbecco.** 1977. Characterization of polyoma virus T antigen. *Proc. Natl. Acad. Sci. USA* **74**:1259–1263.
21. **Ito, Y., N. Spurr, and B. E. Griffin.** 1980. Middle T antigen as primary inducer of full expression of the phenotype of transformation by polyoma virus. *J. Virol.* **35**:219–232.
22. **Lania, L., M. Griffiths, B. Cooke, Y. Ito, and M. Fried.** 1979. Untransformed rat cells containing free and integrated DNA of a polyoma non-transforming (hr-t) mutant. *Cell* **19**:793–802.
23. **Liang, T. J., G. G. Carmichael, and T. L. Benjamin.** 1984. A polyoma mutant that encodes small T antigen but not middle T antigen demonstrates uncoupling of cell surface and cytoskeletal changes associated with cell transformation. *Mol. Cell. Biol.* **4**:2774–2783.
24. **MacPherson, I., and L. Montagnier.** 1964. Agar suspension culture for the selective assay of cells transformed by polyoma virus. *Virology* **23**:291–294.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. *Cell* **33**:153–159.
27. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin.** 1982. The role of individual polyoma virus early proteins in oncogenic transformation. *Nature (London)* **300**:713–718.
28. **Rassoulzadegan, M., Z. Naghashfar, A. Cowie, A. Carr, M. Grisoni, R. Kamen, and F. Cuzin.** 1983. Expression of the large T protein of polyoma virus promotes the establishment in culture of “normal” rodent fibroblast cell lines. *Proc. Natl. Acad. Sci. USA* **80**:4354–4358.
29. **Richardson, W. D., B. L. Roberts, and A. E. Smith.** 1986. Nuclear localization signals in polyoma virus large T antigen. *Cell* **44**:77–85.
30. **Robins, T., C. Jhappan, J. Chirikjan, and G. F. Vande Woude.** 1986. Molecular cloning of the “intronless” EJ ras oncogene using a murine retrovirus shuttle vector. *Gene Analysis Techniques* **3**:12–16.
31. **Rundell, K., E. O. Major, and M. Lampert.** 1981. Association of cellular 56,000- and 32,000-molecular-weight proteins with BK virus and polyoma virus t-antigens. *J. Virol.* **37**:1090–1093.
32. **Schaffhausen, B. S., and T. L. Benjamin.** 1979. Phosphorylation of polyoma T antigen. *Cell* **18**:935–946.
33. **Scher, C. D., R. C. Shepard, H. N. Antoniades, and C. D. Stiles.** 1979. Platelet-derived growth factor and the regulation of the mammalian fibroblast cell cycle. *Biochim. Biophys. Acta* **560**:217–241.
34. **Schlegel, R., and T. Benjamin.** 1978. Cellular alterations dependent upon the polyoma virus hr-t function: separation of mitogenic from transforming capacities. *Cell* **14**:587–599.
35. **Segawa, K., and Y. Ito.** 1982. Differential subcellular localization of *in vivo*-phosphorylated and nonphosphorylated middle-sized tumor antigen of polyoma virus and its relationship to middle-sized tumor antigen phosphorylating activity *in vitro*. *Proc. Natl. Acad. Sci. USA* **79**:6812–6816.
36. **Smart, J. E., and Y. Ito.** 1978. Three species of polyoma virus tumor antigens share common peptides probably near the amino termini of the proteins. *Cell* **15**:1427–1437.
37. **Smith, A. E., R. Smith, B. E. Griffin, and M. Fried.** 1979. Protein kinase activity associated with polyoma virus middle T antigen *in vitro*. *Cell* **18**:915–924.
38. **Soeda, E., J. R. Arrand, N. Smoller, J. E. Walsh, and B. E. Griffin.** 1980. Coding potential and regulatory signals of the polyoma virus genome. *Nature (London)* **283**:445–453.
39. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1**:327–341.
40. **Treisman, R., U. Novak, J. Favaloro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle T protein. *Nature (London)* **292**:595–600.
41. **Tyndall, C., G. L. Mantia, C. M. Thacker, J. Favaloro, and R. Kamen.** 1981. A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. *Nucleic Acids Res.* **9**:6231–6250.
42. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. *Cell* **14**:725–731.
43. **Wulf, E., A. Deboben, F. A. Bautz, H. Faulstich, and T. Wieland.** 1979. Fluorescent phalloidin, a tool for visualization of cellular actin. *Proc. Natl. Acad. Sci. USA* **76**:4498–4502.
44. **Yang, Y.-C., P. Hearing, and K. Rundell.** 1979. Cellular proteins associated with simian virus 40 early gene products in newly infected cells. *J. Virol.* **32**:147–154.
45. **Zhu, Z., G. M. Veldman, A. Cowie, A. Carr, B. Schaffhausen, and R. Kamen.** 1984. Construction and functional characterization of polyomavirus genomes that separately encode the three early proteins. *J. Virol.* **51**:170–180.