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# Tumor induction by a transformation-defective polyoma virus mutant blocked in signaling through Shc

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**ABSTRACT** **Transformation of cells in culture by polyoma virus requires integration of signals downstream of middle T–Shc and middle T-phosphatidylinositol 3-kinase interactions, but the same is not true for induction of tumors in the mouse. Thus, a middle T mutant defective in transformation and blocked in binding Shc is able to induce a broad spectrum of tumors after inoculation into newborn mice. The "tumor profile" induced by the mutant shows enhancement of tumors at some sites and reductions at others but otherwise resembles that induced by the wild-type virus. A nontransforming double-mutant blocked in binding phosphatidylinositol 3-kinase as well as Shc is severely affected but still induces some tumors. These results show that pathways that must cooperate to induce full transformation of cells *in vitro* can act independently and are to a large extent redundant in tumor induction.**

Oncogenic functions of tumor viruses have been identified and characterized largely through the use of *in vitro* cell transformation assays. These assays use fibroblasts almost exclusively as target cells and rely on parameters such as focus formation, growth in soft agar, or growth under limitations of serum as indications of neoplastic change (1). While viral functions associated with transformation generally are assumed to be the same as those required by the virus to induce tumors in an intact host, this may not always be true. For example, the immortalization function of polyoma virus, facilitated by binding of large T antigen to the retinoblastoma gene product, is dispensable for tumor induction (2). Polyoma induces a wide range of epithelial and mesenchymal tumors in mice (3). A number of polyoma virus mutants are available with well characterized defects at the molecular level and with complete or partial defects in transformation. This system therefore should be useful in testing the general validity of *in vitro* transformation as a model of tumor induction and in identifying molecular pathways involved in neoplastic transformation of a variety of cell types *in vivo*.

Middle T is the major transforming protein of polyoma as revealed in various cell transformation assays (4, 5). Its actions are mediated through formation of complexes with $pp60^{c\text{-}src}$. Phosphorylation of middle T on specific tyrosines then promotes binding of several SH2 or phosphotyrosine binding domain-containing proteins involved in mitogenic signaling. These include the Shc adaptor proteins through tyrosine-250 (6, 7), phosphatidylinositol 3-kinase through tyrosine-315 (8–10), and phospholipase C-$\gamma$ through tyrosine-322 (11). Substitution of these tyrosines in middle T by other amino acids leads to functional defects with greater or lesser effects on cell transformation (11–15).

The transforming and tumor-inducing properties of a 315YF mutant have been reported previously. Both properties are affected as seen in the induction of a partially transformed phenotype in established rat fibroblasts (12, 15) and of a delayed and reduced tumor response in mice with altered histological appearance of certain tumors (9, 16). Here we report results of studies on tumor induction by a 250YS mutant and by a 250YS/315YF double mutant. The most remarkable aspect of the results is the retention by the 250YS mutant of a nearly wild-type ability to induce tumors despite its severely compromised ability to transform cells in culture. The double-mutant, blocked in two major signaling pathways and unable to induce either foci or soft agar growth, still induces some tumors, though with reduced frequency and restrictions in cell type.

## MATERIALS AND METHODS

**Viruses and Cells.** The 250YS (tyrosine at position 250 replaced by serine) and 250YS/315YF double mutant were constructed on the PTA wild-type virus background by site-directed mutagenesis as previously described (15). Virus stocks were grown on primary baby mouse kidney epithelial cells and titered by plaque assay on NIH 3T3 cells. Transformation assays were carried out using F111 rat fibroblasts (15).

**Tumor Profiles.** Newborn mice (<18 hr old) of the C3H/BiDa strain were inoculated intraperitoneally with approximately 0.05 ml of crude virus suspension with titers of $2–10 \times 10^6$ plaque-forming units per ml. The animals were inspected twice weekly and followed for development of tumors for up to 14 months. Animals were necropsied when moribund. Grossly normal and tumor tissues were excised and processed for histological examination as previously described (3). The procedure for whole mouse section hybridization also has been described (17).

## RESULTS

**Transformation Defects of Mutants 250YS, 315YF, and 250YS/315YF.** Effects of the single mutations 250YS and 315YF on transformation have been compared using F111 established rat fibroblasts (15). Both mutations lead to transforming defects in which foci appear but with distinctly altered morphologies and at greatly reduced frequencies compared with wild type. The ability of the mutants to induce growth in soft agar is sharply reduced in the case of 315YF and nearly abolished in the case of 250YS. This is illustrated in Fig. 1, which shows cells cloned from foci induced by each of the mutants and by wild-type virus, then expanded and put in soft agar. With respect to morphological criteria and anchorage independent growth, the 250YS mutant is more severely affected than the 315YF mutant. The double-mutant induces no discernible foci on F111 cells and no growth in soft agar, and therefore is considered to be nontransforming.

**Tumor Induction by PTA-250YS.** Effects of the middle T mutations on tumor induction were evaluated by intraperitoneal inoculation of approximately $1–5 \times 10^5$ plaque-forming units of crude virus into newborn C3H/BiDa mice. The mutants were constructed on the PTA "wild-type" virus strain

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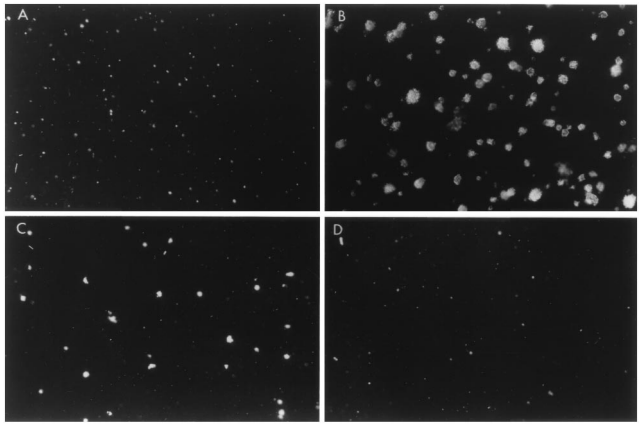


FIG. 1. Agar suspension cultures of normal F111 cells (*A*), F111 transformed by wild-type polyoma virus (*B*), F111 transformed by polyoma mutant 315YF (*C*), and F111 transformed by polyoma mutant 250YS (*D*). Cells in *B*, *C*, and *D* were derived from foci of morphologically transformed cells (see ref. 15).

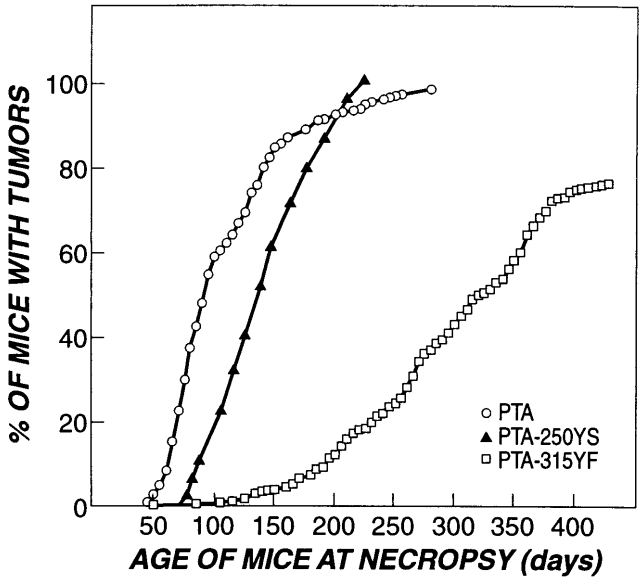


FIG. 2. Cumulative incidence of mice necropsied with tumors after inoculation with wild-type or mutant strains of polyoma virus. Data for PTA and PTA-315YF have been presented previously (16).

background to ensure that coding sequences for VP1 and regulatory sequences were optimal for tumor induction (18–20). Table 1 shows results for PTA-250YS along with those reported earlier for the wild-type and 315YF mutant strains for comparison (16). Data are given for the overall frequency of mice that developed tumor(s), frequencies of individual tumor types, and the average time to necropsy based on developing a moribund condition as a measure of latency. PTA-250YS induced multiple tumors in 100% of the animals and with an average latency only slightly longer (138 days) than for wild-type PTA (108 days). These results contrast with those for PTA-315YF, which showed a reduction in overall tumor frequency and a markedly increased average latency (301 days). This difference in the time required for tumor development is shown graphically in Fig. 2; it represents a striking biological difference between the two mutants.

Despite the overall similarity in tumor induction by PTA and PTA-250YS, some significant differences were noted with respect to frequencies and sizes of individual tumor types. Numerous and extremely large hair follicle tumors were seen in 100% of mutant-infected animals. Microscopic sections of typical mutant and wild-type hair follicle tumors are shown in Fig. 3 *A* and *B*. The tumors induced by PTA-250YS were estimated to be several hundred times larger in volume on average than those induced by the wild-type virus. The opposite tendency was seen with respect to tumors of the thymus. These occurred less frequently in mutant infected mice; in addition, with only one exception, these tumors were detected only microscopically. In contrast, wild-type virus infected mice typically develop large thymic tumors (Fig. 3 *C* and *D*). These tumors grow rapidly and cause respiratory distress, leading to early sacrificing of the animals. The extended latency observed with 250YS (Fig. 2) is most likely due to the failure of this mutant to induce large thymic tumors specifically and not to a slower growth rate of mutant compared with wild-type tumors in general.

Penile papillomas are extremely rare in wild-type-infected animals but were found in about two-thirds of male mice inoculated with PTA-250YS; three pharyngeal papillomas also were noted among the mutant-infected mice. Thyroid tumors were seen microscopically in 19 of the 66 mutant-infected animals. Microscopic ovarian mesotheliomas were seen in almost half of the female mice. Among the males there were two examples of hyperplasia of the prostate and three tumors

Table 1. Tumor profiles induced by wild-type PTA, 315YF, and 250YS mutant viruses

| Tumors | Nos. of mice | | |
|---|---|---|---|
| | PTA | 315YF | 250YS |
| Fraction of mice with tumors(s) | 256/259 (99) | 257/332 (77) | 66/66 (100) |
| Mean age at necropsy in days | 108 | 301 | 138 |
| Epithelial tumors | | | |
| Thymus | 227 (88) | 185 (56) | 45 (68)* |
| Skin: Hair follicle | 229 (88) | 125 (38) | 66 (100) |
| Papilloma | 2 (1)* | 29 (8) | 29 (44) |
| Mammary gland | 126 (49) | 80 (24) | 37 (56) |
| Salivary gland | 148 (57) | 13 (4) | 47 (71) |
| Adrenal medulla | 35 (14) | 0 (0) | 0 (0) |
| Mesenchymal tumors | | | |
| Kidney | 123 (47) | 5 (2) | 5 (8)* |
| Bone | 58 (22) | 130 (39) | 51 (77) |
| Subcutaneous connective tissue | 56 (22) | 12 (4) | 25 (38) |
| Vascular endothelium | 12 (5) | 14 (4) | 5 (8)* |

Numbers indicate the number of mice (% in parentheses) with at least one tumor of the designated type. Data for PTA and 315YF were reported previously (16). Both gross and microscopic tumors are reported.
*Tumors that were detected only or primarily microscopically.

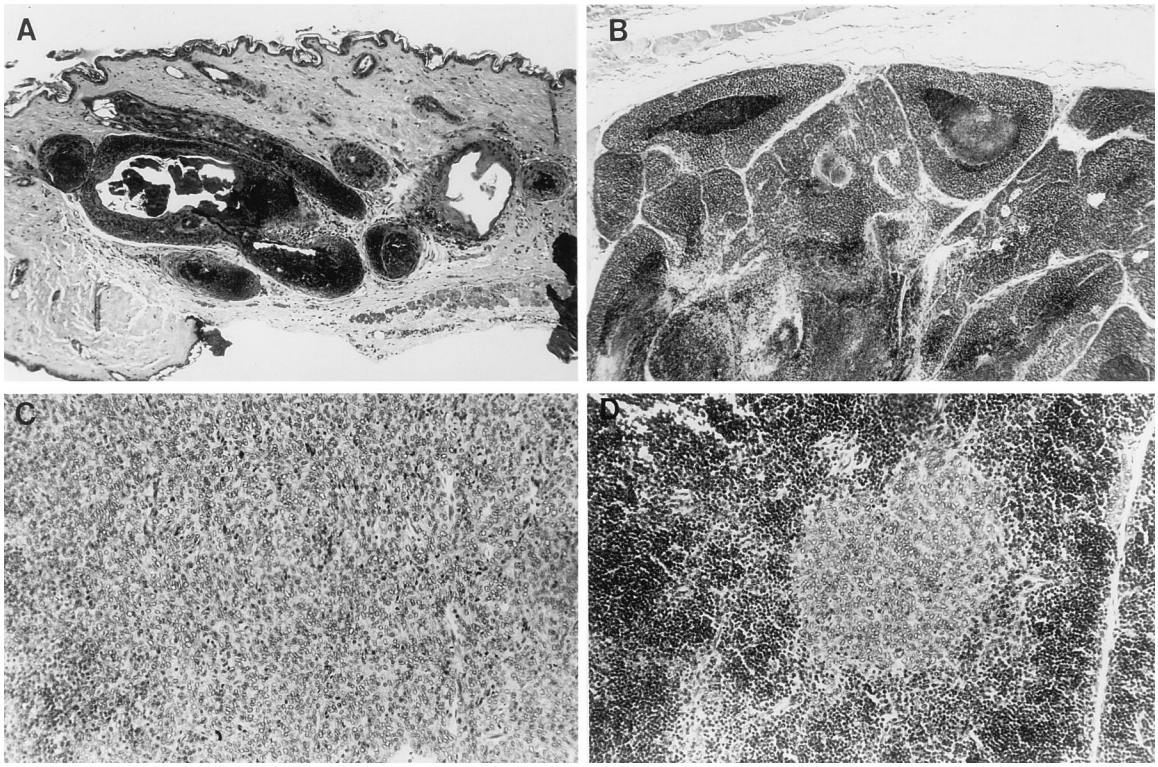


FIG. 3. Microscopic sections of tumors induced by wild-type and mutant polyoma strains. (*A*) Hair follicle tumors induced by wild-type polyoma virus. These tumors typically grow to only several times the diameter of a normal hair follicle (×90). (*B*) Hair follicle tumor induced by polyoma mutant 250YS. At this magnification (×90) only part of the tumor is seen. (*C*) A portion of a thymic epithelioma induced by wild-type polyoma virus. These tumors grow to sizes upwards of 2 cm in diameter (× 180). (*D*) Thymic epithelioma induced by polyoma mutant 250YS. The tumor is histologically identical to the wild-type tumor (*C*) but relatively very small (×180).

of the coagulating gland. These tumor types either have not been seen or seen only very rarely in wild-type-infected animals. Renal sarcomas were induced at a much lower frequency by the 250YS mutant and were seen primarily microscopically. However, other mesenchymal tumors, e.g., in bone or subcutaneous connective tissue, appeared grossly and at somewhat elevated frequencies. No adrenal gland tumors were seen in mice infected by either of the mutant viruses.

**Tumor Induction by PTA-250YS/315YF.** To investigate the extent to which tumor induction by 250YS depends on retention of functions related to binding of phosphatidylinositol 3-kinase, the double mutant PTA-250YS/315YF was constructed and tested (Table 2). Only two small epithelial tumors, one mammary and one thymus, were found among l9 mice inoculated. However, a majority of these animals developed subcutaneous fibrosarcomas or other mesenchymal tumors. No renal sarcomas were found, consistent with the results found for the single mutants (see Table 1). The sharp reduction in overall tumor frequency, increased latency, and preponderance of mesenchymal over epithelial tumors in the double mutant tumor profile represent the major differences from wild type. The residual tumor-inducing ability of this mutant presumably reflects still unaltered aspects of signaling from middle T, for example via binding of phospholipase C-$\gamma$ (11) and elevated production of inositol trisphosphate (21), along with possible contributions from large and small T antigens.

Table 2. Tumor profile induced by PTA-250YS/315YF

| Tumors | No. of mice |
|---|---|
| Fraction of mice with tumor(s) (%) | 15/19 (79) |
| Mean age at necropsy in days | 238 |
| Epithelial tumors | |
| Thymus | 1 (5) |
| Skin: Hair follicle | 0 (0) |
| Papilloma | 0 (0) |
| Mammary gland | 1 (5) |
| Salivary gland | 0 (0) |
| Adrenal medula | 0 (0) |
| Mesenchymal tumors | |
| Kidney | 0 (0) |
| Bone | 3 (16) |
| Subcutaneous connective tissue | 14 (74) |
| Vascular endothelium | 1 (5) |

Numbers indicate the number of mice (% in parentheses) with at least one tumor of the designated type. Both gross and microscopic tumors are reported.

**Virus Replication and Sequence Confirmation.** Mutant and wild-type virus-infected mice were sacrificed at 10 days postinoculation for whole mouse section hybridizations and immunohistochemical staining of kidney sections for viral capsid protein VP1. By these criteria, PTA-250YS replicates and spreads in the neonatally infected mouse essentially as well as wild-type PTA (data not shown).

Viral sequences in tumor and kidney tissue from six mice inoculated with PTA-250YS were amplified by PCR and sequenced to confirm the mutation. All samples yielded the expected mutant sequence, indicating that the tumors were induced by the mutant and not by wild-type virus, which might have arisen by reversion or contamination. The recovered mutant viral DNAs also retained a 40-bp duplication of regulatory sequences around the origin of replication known to be essential in PTA for development of large thymic tumors (19). The reduced frequency and small size of thymic tumors induced by 250YS is therefore due to the loss of middle T-Shc interaction and not to accidental loss of the required regulatory sequences.

## DISCUSSION

Mutations in the NPXY sequence of polyoma middle T lead to loss of transforming ability and loss of binding of the Shc

proteins (6, 7, 13–15, 22). The latter comprise a family of three proteins related by alternative splicing and translational initiation, each containing an N-terminal phosphotyrosine binding domain and a C-terminal SH2 domain (23, 24). Association of Shc with middle T occurs through the phosphotyrosine binding domain of Shc (24) and depends on phosphorylation of tyrosine-250 in the NPXY sequence (6, 7, 15). Shc proteins are important components in mitogenic signaling pathways leading from both receptor and nonreceptor tyrosine kinases through Grb2 and mSos1 to activation of p21 ras (25). As such, they are important in coupling extracellular signals to the mitogen-activated protein kinase pathway and other downstream targets. Overexpression of Shc in established mouse fibroblasts leads to transformation and tumorigenicity in the nude mouse (23). Transformation of established rat fibroblasts to anchorage-independent growth can be mediated through at least two receptor tyrosine kinases in pathways involving the Shc proteins (26, 27).

That the 250YS mutant, blocked in Shc binding, is able to induce a wide array of tumors is surprising. This mutant transforms cells in culture less well than another partially transforming virus mutant 315YF, yet is more tumorigenic, and indeed induces tumors in a manner more closely resembling wild-type virus. The ability of polyoma virus to induce tumors can clearly be dissociated from its ability to induce anchorage independent growth, the criterion usually accepted as the best indication of neoplastic transformation *in vitro*. This dissociation is apparent even with respect to the fibroblast as a common target *in vitro* and *in vivo*, because both 250YS and 250YS/315YF induce fibrosarcomas, yet fail to induce anchorage independent growth.

Although the 250YS mutant remains highly tumorigenic, the loss of middle T-Shc interaction is not without effects in the animal. The 250YS mutation leads to reductions in the frequency and/or size of tumors at some sites such as kidney, thymus, and adrenal gland, and to enhancement at sites such as skin and bone among others. How inactivation of a middle T signaling pathway essential for transformation leads to enhancement of tumor development in certain target cells is not clear. The mutation conceivably could shift the balance away from lytic virus growth thereby increasing the number of viable tumor cells (28), or the pathway itself may generate a negative growth signal in certain types of cells.

It is unlikely that tumor induction by the mutant is due to intrinsic leakiness of the mutation because 250YS and similar mutations effectively abolish Shc binding *in vitro* (6, 7, 15, 29). What appears more likely is that redundant or alternative pathway(s) initiated by the virus can lead to tumor induction in the absence of Shc binding to middle T. Tumor induction by 250YS depends heavily on retention of phosphatidylinositol 3-kinase binding. Thus, introduction of the 315YF mutation on the 250YS mutant background has a far more drastic effect than on the wild-type background. The double-mutant 250YS/315YF induces practically no epithelial tumors, whereas such tumors comprise a majority of those induced by both wild-type and 315YF mutant viruses. Similarly, retention of Shc binding by the 315YF mutant must contribute to the latter's tumor-inducing ability. Middle T-Shc interaction thus operates *in vivo* with broad effects on tumor development despite the fact that absence of such interaction by itself has relatively minor effects.

Important questions remain concerning the downstream targets of middle T's multiple signaling pathways. Two biochemical functions that have been linked to both neoplastic transformation and normal mitogenesis in a variety of systems are elevation of glucose transport and activation of ribosomal protein S6 phosphorylation. In polyoma-infected 3T3 cells, these functions depend entirely on middle T binding to phosphatidylinositol 3-kinase (15, 29). Signaling through phosphatidylinositol 3-kinase and its target serine/threonine kinase Akt has been shown to result in inhibition of apoptosis in several different systems (30–32). Activation of phosphatidylinositol 3-kinase by middle T also appears to block cell death in polyoma virus-infected cells (J. Dahl and T.B., unpublished observation), and this could explain, at least in part, the more rapid growth of wild type and 250YS compared with 315YF tumors. The importance of altered regulation of phosphatidylinositol 3-kinase in oncogenesis by polyoma virus is consistent with the recent finding of an avian retrovirus transducing a catalytic subunit of phosphatidylinositol 3-kinase as its oncogene (P. Vogt, personal communication).

Middle T signaling pathways may not always affect downstream targets in a discrete or exclusive way (33, 34). These pathways may intersect, and the manner of their interaction and consequent effects on cell growth are likely to differ in different cell types. Results in the tumor profiles of the 250YS and 315YF mutants indeed provide evidence for effective redundancy of the pathways in inducing tumors at some sites, for synergism or complementation between the pathways at other sites, and even for negative effects of the pathways resulting in enhancement of tumor induction by the mutants over wild type at still other sites.

**Note:** We have learned of a recent independent study that also shows extensive tumor induction by PTA-250YS (35).

Clyde Dawe died on July 5, l996. We acknowledge this loss of a dear friend and colleague with great sadness. His wisdom and devotion to cancer research will remain an inspiration to all who knew and worked with him. We would like to thank J. Fung and C. Riney for their skillful technical assistance. This work has been supported by a grant from the National Cancer Institute (R35-44343).

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