Recombinant Retroviruses That Transduce Middle T Antigen cDNAs Derived from Polyomavirus Mutants: Separation of Focus Formation and Soft-Agar Growth in Transformation Assays and Correlations with Kinase Activities In Vitro

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To study correlations between cellular transformation and the biochemical properties of polyomavirus middle T antigen, middle T cDNAs have been derived from the polyomavirus mutants *dl1015*, *dl23*, and *NG59b* and have been introduced into rodent fibroblast cell lines by using a retrovirus vector. It was found that all three mutants are completely defective in inducing growth in soft agar but possess a range of activities in assays of focus formation on cell monolayers. Furthermore, when assays of middle T antigen-associated kinase activities were performed in vitro, a correlation between the level of associated phosphatidylinositol kinase activity and the ability of mutant middle T antigens to induce focus formation was observed. However, the association of this activity with middle T antigen does not appear to be sufficient to bring about full transformation, since the middle T antigen derived from *dl1015* is completely defective for soft-agar growth but is associated with a level of phosphatidylinositol kinase activity which is comparable to that of the wild type. Therefore, some other unidentified middle T antigen function may also be required for full transformation.

The middle T antigen (MTAg) of polyomavirus has an acute transforming activity which has been well studied in recent years. An MTAg cDNA can efficiently induce anchorage-independent growth and tumorigenicity when it is introduced by a number of methods into a variety of rodent and avian cell types (7, 13, 24, 25, 39, 40, 47). Genetic studies of polyomavirus transformation have demonstrated the central role which MTAg plays in this process (2, 15, 21). For example, a point mutant of polyomavirus whose only defect is a failure to express MTAg is completely defective for transformation (27).

Studies of the biochemical properties of MTAg have concentrated on its stable association with cellular tyrosine kinases of the *src* family and on the accompanying increase in activity of the complexed cellular enzymes in vitro (4, 8, 11, 12, 26). This association appears to be a necessary step in MTAg transformation, since transforming mutants that are not associated with such activity have never been isolated (reviewed in reference 34). Furthermore, a rat cell line carrying an inducible MTAg gene shows a dose-dependent transformation phenotype that correlates with the level of that fraction of MTAg which is associated with tyrosine kinase activity (39). These results suggest that transformation by MTAg involves modification of the activities of cellular tyrosine kinases, a hypothesis consistent with the general observation that many oncogenes and growth factor receptors encode protein kinases (reviewed in references 3 and 19). However, the association and activation of protein kinase activity that are observed in vitro cannot fully account for the ability of MTAg to induce transformation, since there exist transformation-defective mutants which are associated with wild-type levels of this activity (23, 33, 36).

It has recently been shown that MTAg immunoprecipitates are also associated with an enzymatic activity which

can phosphorylate phosphatidylinositol (PI) in vitro, a property shared with other oncogene products (23, 28, 45, 52). This PI kinase, the type I PI kinase (51), may be an 85-kilodalton tyrosine-phosphorylated protein which is also present in MTAg immunoprecipitates. In vitro, the type I PI kinase makes a novel product, 1,3 PIP. The relevant products of the enzyme in vivo are not known, nor is it known how these products fit into the processes of cellular physiology (50). However, genetic studies of MTAg-associated type I PI kinase activity indicate that it correlates well with transformation competence (9, 22, 23), supporting the hypothesis that PI kinase association, and perhaps its activation by tyrosine phosphorylation, might be necessary steps in transformation by MTAg. The hypothesized role of this PI kinase in the control of proliferation of fibroblasts is further supported by experiments showing that expression of other oncogenes and stimulation with platelet-derived growth factor result in an increase in type I PI kinase activity in immune complexes prepared with an antiphosphotyrosine antiserum (22, 51).

To investigate further the correlations between transformation and the levels of in vitro kinase activities associated with MTAg immunoprecipitates, we have derived MTAg cDNAs from three polyomavirus mutants and have used a retrovirus expression vector to introduce them into rodent cell lines. Using an approach which affords a significant standardization and simplification of the genetic background compared with experiments with polyomavirus mutants, we observed a correlation between the levels of associated PI kinase activity and the ability of the mutant MTAGs to induce focus formation on cell monolayers. However, full transformation resulting in the ability to grow in soft agar appears to require an additional, unidentified MTAG function which might contribute directly to this aspect of the transformed phenotype.

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TABLE 1. Growth in soft agar of NIH 3T3 clones

MTAg	Growth ratio ^a
MT-WT	5/5 ^b
MT-1015	0/4
MT-23	1/4 ^c
MT-59b	0/3
ZIP ^d	1/10 ^b

^a Number of clones that grew per number of clones assayed.^b Growth evident 3 days postseeding.^c Growth evident only after prolonged incubation (2 to 3 weeks).^d G418-resistant clones isolated after infection with control pZIPNeoSV (X)1 retrovirus carrying no insert.

described previously (38). Assays of in vitro protein and phosphatidylinositol kinase activities were performed as described by Kaplan et al. (23). Lysis buffers contained 2 mM sodium orthovanadate. Immunoprecipitations for in vitro assays were performed on extract amounts normalized to give approximately equal amounts of MTA_g as determined by Western blotting.

Western blotting. Western blotting was performed as described previously (35). The same anti-polyomavirus T-antigen antiserum used in immunoprecipitations was diluted 1:1,200 for incubation with blots.

RESULTS

Construction of retrovirus vectors and establishment and titration of psi-2 packaging lines. As summarized in Fig. 1 and in Materials and Methods, MTA_g cDNAs derived from the polyomavirus mutants *dl1015*, *dl23*, and NG59b were constructed and cloned into the pZIPNeoSV(X)1 retrovirus vector. Culture supernatants of psi-2 packaging clones established by selection with G418 after CaPO₄ transfection of plasmid DNAs were found to have titers in the range of 10² to 10⁴ G418-resistant CFU ml⁻¹. To carry out focus formation assays and to establish G418-resistant clones, NIH 3T3, BLAB/3T3, and FR 3T3 cells were infected with retroviruses as described in Materials and Methods.

Transformation phenotypes of mutant MTA_gs. Mutant and wild-type MTA_gs transduced by recombinant retroviruses were assayed for the ability to induce anchorage-independent growth (Table 1). G418-resistant NIH 3T3 clones expressing MTA_gs were seeded in soft agar and scored for growth 4 weeks later. As shown, all mutants were completely defective for soft-agar growth. Whereas five of five wild-type clones grew readily, mutant clones failed to form colonies above the background frequency of slow-growing spontaneous transformants that are occasionally isolated from the parental cells. Similar results were obtained with pooled populations of 50 to 100 G418-resistant NIH 3T3 clones and with individual G418-resistant clones derived from BALB/3T3 cells (data not shown).

Established lines of rodent fibroblasts were infected with recombinant retroviruses to assay for the ability to induce formation of foci on cell monolayers (Fig. 2). When BALB/3T3 cells were infected with amounts of retrovirus that gave comparable numbers of G418-resistant colonies (Fig. 2B), a range of focus-forming activities was observed. After 3 weeks of incubation, it was evident that all mutants were significantly defective in this activity compared with the wild type (Fig. 2A). However, both MT-1015 and MT-23, but not MT-59b, retained some degree of this activity, since both were able to induce formation of slow-growing foci (Fig. 2A and C). Furthermore, it was evident in long-term assays that

MT-1015 retained significantly more focus-forming activity than MT-23 (Fig. 2C). Under these conditions, MT-1015 induced dense focus formation at a frequency comparable to the G418-resistant titer of the retroviral supernatant, whereas MT-23 did not. Similar results were obtained in focus formation assays on NIH 3T3 cells. However, none of the mutants were able to cause formation of foci on monolayers of FR 3T3 cells. The wild-type recombinant retrovirus induced the formation of foci on these cells at a frequency comparable to the G418 titer. However, the wild-type foci were clearly smaller than those that were formed on BALB/3T3 or NIH 3T3 cells (data not shown).

Characterization and kinase activities of mutant MTA_gs. Wild-type and mutant MTA_gs are expressed at readily detectable levels as assayed by immunoprecipitation of [³⁵S]methionine-labeled cell lysates (Fig. 3) and by Western blotting (Fig. 4D). They have gel mobilities identical to those of MTA_gs produced during infection with the mutant and wild-type polyomaviruses (data not shown). Furthermore, in the case of MT-59b, there is an alteration in associated cellular proteins identical to that reported for the NG59 polyomavirus mutant (17, 49; D. Pallas, unpublished results). As assayed by immunoprecipitation and Western blotting, there is clone-to-clone variability in steady-state levels of MTA_g expression. Levels are estimated to vary over a range of fivefold between all MTA_g-expressing clones (three to five NIH 3T3 clones assayed for each allele).

The results of assays of associated kinase activities performed in vitro on a set of NIH 3T3 clones are shown in Fig. 4. Similar results were obtained with BALB/3T3 clones. In these experiments, amounts of MTA_g in immunoprecipitates were matched by using proportions of cell extracts that gave MTA_g bands of approximately equal intensity on Western blots (Fig. 4D). Results of assays of MTA_g phosphorylation are shown in Fig. 4A. The MT-1015 MTA_g is associated with wild-type levels of this activity, while MT-23 is associated with significantly reduced levels. This defect in MT-23 is expected, since tyrosines 315 and 322, two phosphate acceptors in the in vitro reaction, are deleted in the MTA_g encoded by the *dl23* mutant (41, 43) (Fig. 1). The results of protein kinase assays with acid-denatured enolase are shown in Fig. 4B. MT-1015 and MT-23 immunoprecipitates were both associated with levels of this activity that are high and comparable to those of the wild type. In both assays of associated protein kinase activity, MT-59b immunoprecipitates showed no activity above background. Results of assays of PI kinase activity associated with MTA_g immunoprecipitates are shown in Fig. 4C. MT-1015 MTA_g immunoprecipitates phosphorylated PI to a level comparable to that of the wild type, while both the MT-23 and MT-59b gene products were associated with significantly reduced levels of this activity. In these experiments there appeared to be a correlation between levels of associated PI kinase activity and levels of an 85-kilodalton phosphoprotein in the MTA_g immunoprecipitates (Fig. 3 and 4A and C). We and others have hypothesized that this protein is a cellular PI kinase or is closely associated with one (9, 22; Pallas et al., manuscript submitted for publication).

DISCUSSION

We have described an approach to the genetic study of MTA_g that has advantages over the use of mutants of polyomavirus. Sequences encoding MTA_g were effectively isolated from three polyomavirus mutants by recombination in vitro and were introduced into established rodent cell lines

FIG. 2. Focus formation on monolayers of BALB/3T3 cells after infection with mutant and wild-type MTag retroviruses. Cells were divided among three 100-mm-diameter plates after infection. Cultures were incubated in Dulbecco minimal essential medium plus 10% newborn calf serum with (B) and without (A) G418 for 3 weeks or in medium without G418 for 8 weeks (C) and then fixed and stained. WT, MT-WT; 1015, MT-1015; 23, MT-23; 59b, MT-59b.

FIG. 3. [^{35}S]methionine labeling and immunoprecipitation of MTAgs expressed in NIH 3T3 clones. Immunoprecipitations with rabbit anti-polyomavirus T-antigen antiserum were performed on labeled cell lysates normalized for total protein. Proteins were separated by electrophoresis on a 10% polyacrylamide-sodium dodecyl sulfate gel and visualized by fluorography. Mutant and wild-type MTAgs are indicated by arrowheads. The lower band in the MT-WT (WT) lane is the major MTag breakdown product. Gel mobilities of molecular weight markers are shown on the left. Arrows on the right indicate the positions of cellular proteins that show altered association with the MT-59b MTag. The 85-kilodalton phosphoprotein whose presence in immunoprecipitates correlates with PI kinase activity is indicated in the MT-WT lane with an asterisk (9, 22; Pallas et al., submitted). 1015, MT-1015; 23, MT-23; 59b, MT-59b; ZIP, pZIPNeoSV(X)1.

by using a retrovirus expression vector carrying a dominant selectable marker. This method simplifies and standardizes the genetic background in comparative transformation and biochemical studies of MTag by eliminating possible effects of the other T antigens. It has been shown that polyomavirus small T antigen can cooperate with MTag in cellular transformation and tumorigenicity (1, 37, 40) and that large T antigen has an establishment function (48) as well as regulatory effects on early gene transcription. Another advantage of this method is the relatively neutral means of selection afforded by the G418 resistance marker carried on the pZIPNeoSV(X)1 vector. In the past, cell lines have often been isolated by using transformation as a selection criterion (15, 41). This method yields clones which may not be representative of the average transformant, since soft-agar growth or focus formation may depend on especially high levels of mutant MTag expression or on complementary mutations in cellular genes. It should be noted that G418 selection itself may exert some bias through its effects on the physiology of resistant cells. For example, neomycin, an aminoglycosidic antibiotic related to G418, has been shown to inhibit the activity of phospholipase C *in vitro* (14). However, to the best of our knowledge, such a G418-imposed bias has never been reported.

At the levels of expression obtained by using the pZIPNeoSV(X)1 vector, we found that all three mutant

FIG. 4. Assays of kinase activities associated with mutant MTAgs. Immunoprecipitates were prepared from the set of clones used for Fig. 3, and reactions using [γ - ^{32}P]ATP were performed *in vitro* as described previously (23). Relative steady-state levels of MTag expression were determined by Western blotting in pilot experiments, and immunoprecipitations were performed on extract amounts normalized for MTag levels determined in this way. Lanes are labeled as described in the legend to Fig. 3. (A) MTag phosphorylation *in vitro*. (B) Phosphorylation of acid-denatured enolase. Products of reactions were analyzed by electrophoresis on 10% polyacrylamide-sodium dodecyl sulfate gels and visualized by autoradiography. MTAgs and the 85-kilodalton phosphoprotein are indicated. The MT-59b gene product is not labeled in the autophosphorylation reaction. The diffuse band in the MT-23 lane is the antibody heavy chain (D. Pallas, unpublished observations). (C) Phosphorylation of PI. Products of reactions performed *in vitro* were analyzed by thin-layer chromatography and autoradiography as described previously (23). O, origin; PIP, PI phosphate. (D) Western blot using an alkaline phosphatase-conjugated second antibody. Aliquots of cell extracts normalized as for the kinase assays described above were electrophoresed on a 7.5% polyacrylamide-sodium dodecyl sulfate gel and transferred to nitrocellulose for antibody incubations as described previously (35).

MTAgs were completely defective for growth in soft agar but possessed a range of activities in focus formation assays. As expected, MT-59b, derived from the reconstructed hr-t mutant NG-59b (6), was completely defective in both assays. MT-23 and MT-1015 were completely defective for soft-agar growth but retained some transformation activity, since both were able to cause focus formation on cell monolayers. Moreover, MT-1015 retained significantly more of this activity than MT-23 did (Fig. 2C). Our results with MT-1015 are consistent with the marked defect in transformation that has been reported for the *d1*1015 mutant (29, 30). However, in contrast to the significant transforming activity reported for the *d1*23 mutant, which included the ability to induce slow soft-agar growth and tumorigenicity (15, 16, 21, 42), the MTag derived from *d1*23 has only slight transformation activity. It is possible that small or large T antigen is able to complement the MTag defect in *d1*23 transformation. As an example of such cooperativity, it has been shown that small

T antigen can complement MTA_g in cells refractory to transformation by the latter alone (37). Another possibility is that MT-23 clones, which were isolated by G418 resistance, express less MTA_g than the stable *dl*23 transformants described in other reports, which were necessarily isolated as foci or as soft-agar colonies.

We observed in the phenotype of the MT-1015 mutant a separation of focus formation and soft-agar growth. This suggests the possibility that the MTA_g function or functions defective in this mutant may be responsible for inducing anchorage-independent growth, while other functions responsible for the ability to form foci on cell monolayers remain intact. However, an interpretation which assigns separate functions for different aspects of transformation in this way may be too simplistic for several reasons. In the case of MT-1015, this separation is not complete, since this mutant showed a marked defect in focus formation compared with the wild type. Furthermore, in experiments in which wild-type MTA_g is expressed from an inducible promoter, a hierarchy of transformation phenotypes which is dependent on the level of oncogene expression is observed (39). At intermediate levels of expression, the cells are able to form foci but are not able to grow in soft agar, while at high levels of expression, both focus formation and soft-agar growth are observed. Finally, though it has been possible to isolate mutants of MTA_g and SV40 large T antigen (E. Paucha, personal communication) which induce focus formation in the absence of soft-agar growth, mutants of any oncogene which retain soft-agar growth in the absence of focus formation have not, to our knowledge, been reported. The simplest interpretation of all these observations is that there is an overlap of functions for focus formation and soft-agar growth in the transformed phenotype and that the threshold requirement for the level of this activity for soft-agar growth is higher than that for focus formation. A prediction of this hypothesis is that at high enough levels of expression, MT-1015 would also induce anchorage-independent growth.

Mutant MTA_gs encoded by the retroviruses were found to have the same biochemical properties reported for MTA_gs produced during infection with the corresponding polyomavirus mutants, indicating that the plasmid DNA constructions are correct and carry the intended lesions. Mutant MTA_gs are expressed at high levels with correct gel mobilities and with previously reported alterations in associated cellular proteins. In addition, mutant gene products show expected levels of associated tyrosine kinase activity in vitro (23). In particular, assays of this activity using an exogenous protein substrate demonstrate that both MT-1015 and MT-23 are associated with levels of protein kinase activity that are comparable to that of the wild type. In assays of associated PI kinase activity, the mutant MTA_gs derived from *dl*23 and NG59b both show marked defects, which is consistent with other reports (9, 22, 23). However, unlike the results of our previous measurements on MTA_g expressed after infection with the *dl*1015 mutant polyomavirus (23), we observed levels of PI kinase activity associated with the MT-1015 MTA_g expressed in stable cell lines which were high and comparable to that of the wild type. Similar results with stable cell lines expressing *dl*1015 viral DNA have been reported by others (9). The reason for this difference between levels of associated PI kinase activity in stable cell lines and virus-infected cells is not known.

The purpose of the experiments described in this report was to investigate correlations between the transformation competence of the mutant MTA_gs and their associated

TABLE 2. Summary of transformation and in vitro kinase results

MTA _g	Transformation ability		In vitro kinase activity	
	Soft-agar growth	Focus formation	PI	Enolase
MT-WT	+	+	+	+
MT-1015	—	+/- ^a	+	+
MT-23	—	— ^b	— ^c	+
MT-59b	—	—	—	—

^a Slow-growing foci on BALB/3T3 and NIH 3T3 cells; no discernible activity on FR 3T3 cells.

^b Slight activity on BALB/3T3 cells; no discernible activity on FR 3T3 cells.

^c Slight activity (<1/10 that of the wild type).

kinase activities in vitro. These results are summarized in Table 2. MT-23, which retains only residual transformation ability, has a level of associated tyrosine kinase activity comparable to that of the wild type but a significantly reduced level of PI kinase activity. MT-1015, the only mutant which demonstrates significant focus-forming ability, is likewise the only mutant whose gene product is associated with a high level of PI kinase activity. Therefore, there appears to be a correlation between association of PI kinase activity and the ability of the MTA_g mutants to induce formation of foci on cell monolayers. However, the high level of PI kinase activity associated with the MT-1015 MTA_g does not correlate with the complete inability of this mutant to induce growth in soft agar nor with its marked defect in focus formation compared with the wild type. This suggests that the association of PI kinase activity with MTA_g that is observed in vitro may not be sufficient in itself to bring about full transformation and that therefore another MTA_g function, which is defective in the MT-1015 mutant, may also be required.

At this time one can only conjecture as to the nature of the functions defective in the MT-23 and MT-1015 mutants. One possibility is that the complex that forms between the *c-src* and/or *c-yes* gene products and the mutant MTA_gs fails to phosphorylate some or all of its normal substrates in vivo, even though it can phosphorylate enolase in immune complexes in vitro. The 85-kilodalton putative type I PI kinase is likely to be such a substrate in the case of MT-23. In any event, identification of the functions defective in these mutants should contribute to our understanding of transformation by polyomavirus MTA_g, and our current efforts are therefore directed towards this problem.

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LITERATURE CITED

- Asselin, C., C. Gelin, 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.
- Benjamin, T. L. 1970. Host range mutants of polyoma virus. *Proc. Natl. Acad. Sci. USA* 67:394-399.
- Bishop, J. M. 1985. Viral oncogenes. *Cell* 42:23-38.
- 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.
5. Cepko, C. L., B. E. Roberts, and R. C. Mulligan. 1984. Construction and applications of a highly transmissible murine retrovirus shuttle vector. *Cell* 37:1053-1062.
 6. Cheng, S. H., W. Markland, A. F. Markham, and A. E. Smith. 1986. Mutations around the NG59 lesion indicate an active association of polyoma virus middle-T antigen with pp60^{c-src} is required for cell transformation. *EMBO J.* 5:325-334.
 7. Cherington, V., B. Morgan, B. M. Spiegelman, and T. M. Roberts. 1986. Recombinant retroviruses that transduce individual polyoma tumor antigens: effects on growth and differentiation. *Proc. Natl. Acad. Sci. USA* 83:4307-4311.
 8. Courtneidge, S. A. 1985. Activation of the pp60^{c-src} kinase by middle T antigen binding or by dephosphorylation. *EMBO J.* 4:1471-1477.
 9. Courtneidge, S. A., and A. Heber. 1987. An 81 kd protein complexed with middle T antigen and pp60^{c-src}: a possible phosphatidylinositol kinase. *Cell* 50:1031-1037.
 10. Courtneidge, S. A., B. A. Oostra, and A. E. Smith. 1984. Tyrosine phosphorylation and polyoma virus middle T protein, p. 123-131. In G. F. Vande Woude, A. J. Levine, W. C. Topp, and J. D. Watson (ed.), *Cancer cells 2. Oncogenes and viral genes*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 11. 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.
 12. Courtneidge, S. A., and A. E. Smith. 1985. The complex of polyoma virus middle T antigen and pp60^{c-src}. *EMBO J.* 3:585-591.
 13. Donoghue, D. J., C. Anderson, T. Hunter, and P. L. Kaplan. 1984. Transmission of the polyoma virus middle T gene as the oncogene of a murine retrovirus. *Nature (London)* 308:748-750.
 14. Downes, C. P., and R. H. Michell. 1981. The polyphosphoinositide phosphodiesterase of erythrocyte membranes. *Biochem. J.* 198:133-140.
 15. Griffin, B. E., Y. Ito, U. Novak, N. Spurr, S. Dilworth, N. Smolar, R. Pollack, K. Smith, and D. B. Rifkin. 1980. Early mutants of polyoma virus (dl8 and dl23) with altered transformation properties: is polyoma virus middle T antigen a transforming gene product? *Cold Spring Harbor Symp. Quant. Biol.* 44:271-282.
 16. Griffin, B. E., and C. Maddock. 1979. New classes of viable deletion mutants in the early region of polyoma virus. *J. Virol.* 31:645-656.
 17. Grussenmeyer, T., K. H. Scheidtmann, M. A. Hutchinson, W. Eckhart, and G. Walter. 1985. Complexes of polyoma virus medium T antigen and cellular proteins. *Proc. Natl. Acad. Sci. USA* 82:7952-7954.
 18. Harvey, R., B. A. Oostra, G. J. Belsham, P. Gillett, and A. E. Smith. 1984. An antibody to a synthetic peptide recognizes polyomavirus middle-T antigen and reveals multiple in vitro tyrosine phosphorylation sites. *Mol. Cell. Biol.* 4:1334-1342.
 19. Hunter, T. 1987. A thousand and one protein kinases. *Cell* 50:823-829.
 20. Hunter, T., M. A. Hutchinson, and W. Eckhart. 1984. Polyoma middle-sized T antigen can be phosphorylated on tyrosine at multiple sites *in vitro*. *EMBO J.* 3:73-80.
 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. Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M. White, L. Cantley, and T. M. Roberts. 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. *Cell* 50:1021-1029.
 23. Kaplan, D. R., M. Whitman, B. Schaffhausen, L. Raptis, R. L. Garcea, D. Pallas, T. M. Roberts, and L. Cantley. 1986. Phosphatidylinositol metabolism and polyoma-mediated transformation. *Proc. Natl. Acad. Sci. USA* 83:3624-3628.
 24. Kaplan, P. L., S. Simon, and W. Eckhart. 1985. Polyomavirus middle T protein encoded by a retrovirus transforms nonestablished chicken embryo cells. *J. Virol.* 56:1023-1025.
 25. Kornbluth, S., F. R. Cross, M. Harbison, and H. Hanafusa. 1986. Transformation of chicken embryo fibroblasts and tumor induction by the middle T antigen of polyomavirus carried in an avian retroviral vector. *Mol. Cell. Biol.* 6:1545-1551.
 26. Kornbluth, S., M. Sudol, and H. Hanafusa. 1987. Association of the polyomavirus middle-T antigen with c-yes protein. *Nature (London)* 325:171-173.
 27. 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.
 28. Macara, I. G., G. V. Marinetti, and P. C. Balduzzi. 1984. Transforming protein of avian sarcoma virus UR2 is associated with phosphatidylinositol kinase activity: possible role in tumorigenesis. *Proc. Natl. Acad. Sci. USA* 81:2728-2732.
 29. Magnusson, G., and P. Berg. 1979. Construction and analysis of viable deletion mutants of polyoma virus. *J. Virol.* 32:523-529.
 30. Magnusson, G., M.-G. Nilsson, S. M. Dilworth, and N. Smolar. 1981. Characterization of polyoma mutants with altered middle and large T-antigens. *J. Virol.* 39:673-683.
 31. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 32. 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.
 33. Markland, W., S. H. Cheng, B. A. Oostra, and A. E. Smith. 1986. In vitro mutagenesis of the putative membrane-binding domain of polyomavirus middle-T antigen. *J. Virol.* 59:82-89.
 34. Markland, W., and A. E. Smith. 1987. Mutants of polyomavirus middle-T antigen. *Biochim. Biophys. Acta* 907:299-321.
 35. Morrison, D. K., P. J. Browning, M. F. White, and T. M. Roberts. 1988. Tyrosine phosphorylations in vivo associated with v-fms transformation. *Mol. Cell. Biol.* 8:176-185.
 36. Nilsson, S. V., C. Tyndall, and G. Magnusson. 1983. Deletion mapping of a short polyoma virus middle T antigen segment important for transformation. *J. Virol.* 46:284-287.
 37. Noda, T., M. Satake, Y. Yamaguchi, and Y. Ito. 1987. Cooperation of middle and small T antigens of polyomavirus in transformation of established fibroblast and epithelial cell lines. *J. Virol.* 61:2253-2263.
 38. Pallas, D. C., C. Schley, M. Mahoney, E. Harlow, B. S. Schaffhausen, and T. M. Roberts. 1986. Polyomavirus small T antigen: overproduction in bacteria, purification, and utilization for monoclonal and polyclonal antibody production. *J. Virol.* 60:1075-1084.
 39. Raptis, L., H. Lamfrom, and T. L. Benjamin. 1985. Regulation of cellular phenotype and expression of polyomavirus middle T antigen in rat fibroblasts. *Mol. Cell. Biol.* 5:2476-2486.
 40. Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin. 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. *Nature (London)* 300:713-718.
 41. Schaffhausen, B. S., and T. L. Benjamin. 1981. Comparison of phosphorylation of two polyomavirus middle T antigens in vivo and in vitro. *J. Virol.* 40:184-196.
 42. Seif, R. 1980. Polyoma virus middle t antigen: a tumor progression factor. *J. Virol.* 35:479-487.
 43. Smolar, N., and B. E. Griffin. 1981. DNA sequences of polyoma virus early deletion mutants. *J. Virol.* 38:958-967.
 44. Soeda, E., J. R. Arrand, N. Smolar, J. E. Walsh, and B. E. Griffin. 1980. Coding potential and regulatory signals of the polyoma virus genome. *Nature (London)* 283:445-453.
 45. Sugimoto, Y., M. Whitman, L. C. Cantley, and R. L. Erikson. 1984. Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. *Proc. Natl. Acad. Sci. USA* 81:2117-2121.
 46. Tooze, J. (ed.). 1981. *Molecular biology of tumor viruses*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 47. 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.
48. **Valle, G. D., R. G. Fenton, and C. Basilico.** 1981. Polyoma large T antigen regulates the integration of viral DNA sequences into the genome of transformed cells. *Cell* **22**:347–355.
49. **Walter, G., A. Carbone, and W. J. Welch.** 1987. Medium tumor antigen of polyomavirus transformation-defective mutant NG59 is associated with 73-kilodalton heat shock protein. *J. Virol.* **61**:405–410.
50. **Whitman, M., L. Fleischman, S. B. Chahwala, L. Cantley, and P. Rosoff.** 1986. Phosphoinositides, mitogenesis, and oncogenesis, p. 197–217. *In* J. W. Putney (ed.), *PI turnover and receptor function*. Alan R. Liss, New York.
51. **Whitman, M., D. Kaplan, T. Roberts, and L. Cantley.** 1987. Evidence for two distinct phosphatidylinositol kinases in fibroblasts: implications for cellular regulation. *Biochem. J.* **247**:165–174.
52. **Whitman, M., D. R. Kaplan, B. Schaffhausen, L. Cantley, and T. M. Roberts.** 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. *Nature (London)* **315**:239–242.