JOURNAL OF VIROLOGY, Apr. 1993, p. 1945–1952
0022-538X/93/041945-08$02.00/0

Vol. 67, No. 4

Mutation of a Cysteine Residue in Polyomavirus Middle T Antigen Abolishes Interactions with Protein Phosphatase 2A, pp60$^{c\text{-}src}$, and Phosphatidylinositol-3 Kinase, Activation of c-*fos* Expression, and Cellular Transformation

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Received 28 September 1992/Accepted 21 December 1992

Polyomavirus middle T antigen (MT) interacts with several cellular proteins involved in cell proliferation. MT forms complexes with protein phosphatase 2A (PP2A), pp60$^{c\text{-}src}$ (and the related kinases c-*fyn* and c-*yes*), and phosphatidylinositol-3 kinase. We made a single point mutation in MT, changing a conserved cysteine residue at position 120 to tryptophan, and characterized the biochemical and biological properties of the mutant (C120W) protein. The mutant MT protein does not associate with PP2A, pp60$^{c\text{-}src}$, or phosphatidylinositol-3 kinase as judged by coimmunoprecipitation and associated phosphatase or kinase activity. The C120W mutant is defective in activation of c-*fos* expression and in morphological transformation of NIH 3T3 cells.

The DNA tumor viruses have provided useful model systems for studies of cellular proliferation and transformation. Polyomavirus encodes three proteins, or tumor antigens, expressed at early times after infection. Large T antigen (LT) is required for viral DNA replication and can immortalize primary cells in vitro (1, 15, 34, 49). Middle T antigen (MT) can induce morphological transformation of established cell lines (20, 58). In productive infection MT enhances the packaging of viral DNA, presumably by affecting the phosphorylation state of VP1, the major capsid protein (26). Small t antigen (st) enhances viral DNA replication (2, 26, 43, 56) and can induce established cells to proliferate to a higher saturation density (9, 33).

These three proteins accomplish their tasks, at least in part, through interactions with host cellular proteins. LT interacts with pRb, the retinoblastoma susceptibility gene product, through a region homologous to those identified in simian virus 40 (SV40) LT, adenovirus E1A, and the human papillomavirus E7 proteins (16, 22, 23, 62). This interaction is required for LT to immortalize primary cells (40).

MT interacts with several cellular proteins, including pp60$^{c\text{-}src}$ (5, 14) and the related kinases c-*yes* and c-*fyn* (8, 32, 38, 39). MT enhances the kinase activity of pp60$^{c\text{-}src}$ (12), which correlates with the dephosphorylation of tyrosine 527 in pp60$^{c\text{-}src}$. MT also has an associated phosphatidylinositol kinase activity (61). MT phosphorylated on Tyr-315 interacts with p85 (13, 36), which has been identified as a subunit of phosphatidylinositol-3 (PI-3) kinase (24, 44). This interaction leads to an increase in PI-3 kinase activity. The interaction between MT and pp60$^{c\text{-}src}$ appears to be necessary but not sufficient for morphological transformation of cells (42). Likewise, the interaction with PI-3 kinase appears to be necessary but not sufficient for transformation (21). MT also interacts with two proteins of 60 to 64 kDa and 37 kDa (30, 31). These have been identified as the regulatory and catalytic subunits of protein phosphatase 2A (PP2A) (47, 60). Association of MT with these two proteins appears to be important for transformation (31, 45, 52); however, the precise role of this association in cellular transformation is unclear, as mutants of MT either interact with both PP2A and pp60$^{c\text{-}src}$ (e.g., mutant 1178T) or interact with neither protein (e.g., SD15 and NG59). PP2A also associates with small t antigens of polyomavirus, SV40, and BK virus (45, 51, 59, 65).

PP2A is an abundant serine/threonine protein phosphatase. It consists of a 37-kDa catalytic subunit (C) and a 60- to 64-kDa regulatory subunit (A). In addition, it may include another regulatory subunit of either 54 kDa (B′), 55 kDa (B), or 72 kDa (B″) (10). SV40 st apparently associates directly with the A subunit, since it can associate with the AC form or the free A subunit but not with the free C subunit or the ABC form of PP2A (64). Therefore it has been suggested that SV40 st acts analogously to the B subunit of PP2A (47, 48, 64).

We report here the construction and characterization of a mutant of polyomavirus MT which does not associate with PP2A. Mutation of a cysteine residue (amino acid 120) in MT results in a protein which can no longer associate with PP2A as judged by coimmunoprecipitation from cells expressing either wild-type MT or the C120W mutant protein. Wild-type MT, but not the C120W protein, has associated phosphatase activity in vitro. The mutant protein also lacks an associated protein kinase activity and PI-3 kinase activity. Wild-type MT, but not the C120W protein, can activate expression from the c-*fos* promoter in transfected cells. Finally, the C120W mutant is defective for the morphological transformation of NIH 3T3 cells.

MATERIALS AND METHODS

Cell lines. BALB/c 3T3 cells and the ecotropic packaging cell line ψ-2 were grown in Cellgro Dulbecco's modified Eagle's medium (DME; Mediatech, Washington, D.C.) supplemented with 10% calf serum. NIH 3T3 cells were ob-

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tained from Marguerite Vogt, Salk Institute, and were also maintained in DME plus 10% calf serum.

Plasmids. pRSV, an expression vector in which a foreign gene is expressed from the Rous sarcoma virus long terminal repeat (19), was obtained from Steve Gould, University of California, San Diego. pRSVMT and pRSVMTC120W contain the coding region of wild-type or mutant MT, respectively, cloned into pRSV. pSLXCMV, a retrovirus expression vector in which the cloned gene is expressed from the cytomegalovirus promoter (53), was obtained from Raphael Scharfmann, Salk Institute. pSLXMT and pSLXMTC120W contain the wild-type and mutant MT coding regions, respectively, cloned into pSLXCMV. pneoMLV, a retrovirus vector based on Moloney murine leukemia virus (37), was used to clone pMTneoMLV (6) and pMTC120WneoMLV. FC-2 is a plasmid in which the bacterial chloramphenicol acetyltransferase gene (CAT) is expressed from the murine c-*fos* promoter (18). pGem4 was obtained from Promega, Madison, Wis.

Immunoprecipitations. Cell lines were seeded at 2×10^5 cells per 6-cm dish for ^{35}S metabolic labeling. After 24 h, cells were labeled for 16 h in 2.5 ml of DME without cysteine or methionine but supplemented with 4% dialyzed calf serum by using 250 μCi of ^{35}S Express label (Du Pont NEN, Boston, Mass.). Cells were washed twice with Tris-buffered saline (TBS), and cell lysates were prepared with Nonidet P-40 (NP-40) lysis buffer (20 mM Tris-HCl [pH 8], 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol [DTT] 20 μg of leupeptin per ml, 1% Trasylol [Mobay Corp., New York, N.Y.], 100 μM Na_3VO_4) and precleared with boiled *Staphylococcus aureus* (Pansorbin; Calbiochem, San Diego, Calif.). Cleared lysates were immunoprecipitated by using a polyomavirus antitumor ascites serum and immune complexes collected with recombinant protein A immobilized on Sepharose beads (Repligen, Cambridge, Mass.) and washed five times with NP-40 buffer. Immunoprecipitates were fractionated on a sodium dodecyl sulfate (SDS)–7% polyacrylamide gel (acrylamide/bisacrylamide ratio, 20:1).

Protein phosphatase assays. Cell lines were seeded at 6×10^5 cells per 6-cm dish. After 24 h, NP-40 lysates were prepared. Immunoprecipitations were carried out as described above; immune complexes were washed three times with NP-40 lysis buffer, twice with Tris buffer (20 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM DTT, 100 μM Na_3VO_4), and once with phosphatase buffer without $MnCl_2$ (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7], 1 mM DTT, 100 μg of bovine serum albumin [BSA] per ml, 50 μM leupeptin, 0.05% NP-40). Washed immunoprecipitates were resuspended in 50 μl of phosphatase buffer containing 1 mM $MnCl_2$, and 50,000 cpm of ^{32}P-labeled casein was added as a substrate. Reactions were carried out at 30°C for 15 min and then terminated by the addition of 50 μl of 1% BSA and 500 μl of 10% trichloroacetic acid. Samples were incubated for 10 min on ice and then centrifuged in a TOMY MC-150 tabletop centrifuge (Peninsula Laboratories, Inc., Belmont, Calif.) Supernatants were counted by Cerenkov counting, using an LS-233 liquid scintillation counter (Beckman, Fullerton, Calif.) to determine counts released. Casein (8 μg; Sigma, St. Louis, Mo.; prepared by Ushio Kikkawa) was phosphorylated for 30 min at 30°C with cyclic AMP (cAMP)-dependent protein kinase (0.42 μg; catalytic subunit purified from rat brain; kindly provided by Ushio Kikkawa) in kinase buffer (20 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$) containing 100 μCi of [$\gamma^{32}P$]ATP and 5 μM nonradioactive ATP in 25 μl. The kinase was inactivated by incubation at 68°C for 10 min; the reaction mixture was brought up to 100 μl with phosphatase buffer, and the casein was purified through three sequential columns of Sephadex G-50 equilibrated in phosphatase buffer.

In vitro kinase assays. Cell lines were seeded at 3×10^5 cells per 6-cm dish. After 24 h, NP-40 lysates (containing 1 mM DTT) were prepared, and immunoprecipitations were carried out as described above, the last wash being done in kinase buffer without $MnCl_2$ [20 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 1 mM DTT]. Immune complexes were resuspended in 25 μl of kinase buffer containing 10 mM $MnCl_2$ and 20 μCi of [$\gamma^{32}P$]ATP, either with or without 20 μM nonradioactive ATP present, and incubated at 30°C for 15 min. The reaction was stopped by adding 1 ml of NP-40 lysis buffer. After centrifugation and removal of the supernatant, samples were resuspended in 2× sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 40% 2-mercaptoethanol, 20 μg of bromophenol blue per ml) and fractionated by SDS-polyacrylamide gel electrophoresis. Enolase was used as an exogenous substrate as described previously (11).

PI-3 kinase assays. Cell lines were seeded at 8×10^5 cells per 10-cm dish. After 18 h, cell lysates were prepared by using 137 mM NaCl, 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 20 μg of leupeptin per ml. Lysates were precleared and immunoprecipitations were carried out with antitumor serum as described above. After incubation with Repligen, immune complexes were collected and washed once with ice-cold phosphate-buffered saline, twice with 0.5 M LiCl–0.1 M Tris (pH 7.4), and once with 10 mM Tris (pH 7.4)–100 mM NaCl–1 mM EDTA as described by Whitman et al. (61). Samples were resuspended in 25 μl of 2× kinase buffer (20 mM HEPES [pH 7.1], 0.8 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid [EGTA], 0.8 mM sodium phosphate)–9.5 μl of 20 mM HEPES (pH 7.1)–0.5 μl phosphatidylinositol (20 mg/ml in dimethyl sulfoxide) and incubated on ice for 20 min. Reactions were started by the addition of nonradioactive ATP, $MgCl_2$ (final concentrations, 40 μM and 10 mM, respectively), and 20 μCi of [γ-^{32}P]ATP in a total volume of 50 μl. Reactions were incubated at 25°C for 20 min and terminated by the addition of 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$-CH_3OH (MeOH) (1:1). The organic phase was washed once with 80 μl of MeOH-HCl (1:1), dried in a Speedy-vac, and spotted in $CHCl_3$-MeOH (4:1) onto a silica gel H thin-layer chromatography plate with 1% potassium oxalate (Analtech, Newark, Del.) which had been baked in a 110°C oven for 30 min. Reaction products were separated by thin-layer chromatography in $CHCl_3$–MeOH–4 M NH_4OH (9:7:2) for 1 h and visualized by autoradiography. The plates were then scanned and quantitated by using a Molecular Dynamics Phosphorimager.

Transfections and CAT assays. BALB/c 3T3 cells were seeded at 2×10^5 cells per 6-cm dish 24 h prior to transfection. Cells were transfected by the modified calcium phosphate-mediated precipitation procedure with BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid]-buffered saline precipitation (7). Then 2 μg of FC-2 was cotransfected with 0.5 μg of either pRSV, pRSVMT, or pRSVC120W, along with 1 μg of pRSVβGal, and 11.5 μg of pGem4. After 14 to 18 h, cells were washed with TBS and refed with DME containing 0.5% calf serum. After 48 h, extracts were prepared, protein determinations were carried out with the BCA reagent (Pierce, Rockford, Ill.), and β-galactosidase and CAT assays were carried out by standard protocols.

FIG. 1. Schematic representation of the conserved cysteine clusters (in bold type) common to MT and st. The mutation made in polyomavirus (PyV) MT, changing cysteine 120 to tryptophan, is indicated.

Transformation assays. The ecotropic retrovirus-packaging cell line ψ-2 was seeded at 3×10^5 cells per 10-cm dish. After 24 h, the cells were transfected with 15 μg of either pneoMLV, pMTneoMLV, or pMTC120WneoMLV by calcium phosphate-mediated precipitation. After 14 to 18 h, the cells were washed with TBS and refed with DME containing 10% calf serum. NIH 3T3 clone 2B cells were then seeded at 5×10^4 cells per 6-cm dish. After 24 h, these cells were infected with supernatants from the transiently transfected ψ-2 cells. Supernatants were filtered through a Millipore filter (pore size, 0.45 μm). The cells were infected with 1 ml of virus plus 1 ml of DME containing 10% calf serum plus 8 μg of Polybrene per ml; six plates of cells were infected with each dilution of virus. The next day, 3 ml of DME containing 1.6% calf serum (final concentration, 5%) was added to three dishes of each dilution for focus assays, and 3 ml of DME containing 10% calf serum was added to the remaining dishes; after 24 h, G418 (Geneticin; GIBCO, Grand Island, N.Y.) was added to these plates (400 μg/ml) to determine the G418-resistant (virus) titer. When foci were visible by eye (14 to 18 days), cells were fixed and stained with crystal violet. Individual colonies as well as mass cultures were obtained from one set of the G418 titration plates to examine protein expression.

RESULTS

Construction and expression of a cysteine mutant of MT. The region in common between polyomavirus MT and st contains a cluster of cysteine residues, which is conserved among small t antigens of several papovaviruses (amino acids 120 to 153 in polyomavirus [Fig. 1]). We used site-directed mutagenesis to change codon 120 from TGT to TGG, thus changing cysteine 120 to tryptophan (Fig. 1). A corresponding mutation was previously shown to abrogate the ability of SV40 st to cooperate with LT to transform BALB/c 3T3 cells (4). This mutant, designated MTC120W, was cloned into several vectors for expression studies (see Materials and Methods).

Cell lines which stably express either the wild-type or the C120W mutant MT proteins were constructed. ψ-2 cells were transiently transfected with the two expression vectors, pSLXMT and pSLXC120W, and their supernatants were used to infect NIH 3T3 cells, which were then selected for G418 resistance. In parallel, a control cell line was constructed by using the expression vector pSLXCMV alone. In all cases, pools of G418-resistant colonies were obtained. Cell lines were monitored for the expression of the expected proteins by immunoprecipitation from lysates labeled with [^{35}S]methionine and cysteine by using polyoma antitumor serum, followed by SDS-polyacrylamide gel electrophoresis, fluorography, and autoradiography. As shown in Fig. 2, lane 2, the cell line NSLXMT expressed a 56-kDa protein corresponding to MT which was not expressed in the control cell line NSLX (Fig. 2, lane 1). The cell line NSLXMTC120W also expressed a similar-sized 56-kDa protein, as would be expected for a missense mutant (Fig. 2, lane 3). The sizes of the expected proteins were confirmed by in vitro transcription and translation (27).

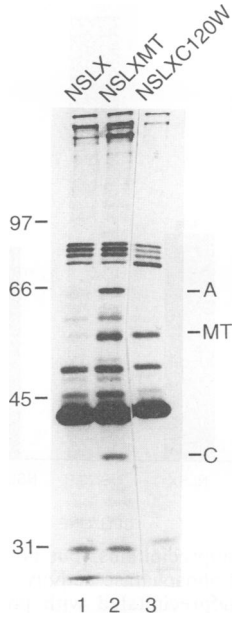

FIG. 2. Association of MT but not MTC120W with PP2A. Cell lines were labeled with ^{35}S-Express label, and NP-40 lysates were immunoprecipitated with polyomavirus antitumor serum. Shown is an autoradiogram of a polyacrylamide gel. Lanes: 1, NSLX cells; 2, NSLXMT cells; 3, NSLXC120W cells. The positions of MT and the regulatory (A) and catalytic (C) subunits of PP2A are indicated.

MTC120W does not associate with PP2A. MT associates with several cellular proteins, notably with pp60$^{c\text{-}src}$, the related kinases c-*yes* and c-*fyn*, PI-3 kinase, and PP2A. It was of interest, therefore, to determine whether the C120W mutant MT protein was altered in its association with any of these proteins. As shown in Fig. 2, MT coimmunoprecipitated two proteins of approximately 60 and 37 kDa (lane 2). The 60- and 37-kDa proteins correspond to the regulatory (A) and catalytic (C) subunits of PP2A, respectively (47, 60). These proteins were not precipitated from control cells (Fig. 2, lane 1). Notably, in the cell line NSLXMTC120W, the mutant MT protein did not associate with these two proteins as judged by the lack of these proteins in the mutant MT immunoprecipitate (Fig. 2, lane 3). Thus it appears that the mutant MTC120W does not associate with PP2A.

MTC120W does not possess an associated phosphatase activity. Since the mutant MT protein, MTC120W, does not associate with PP2A as judged by coimmunoprecipitation, we confirmed and extended this observation by examining the wild-type and mutant MT for associated phosphatase activity. NP-40 lysates of each of the cell lines were prepared and subjected to immunoprecipitation with polyomavirus antitumor serum. The washed immunoprecipitates were

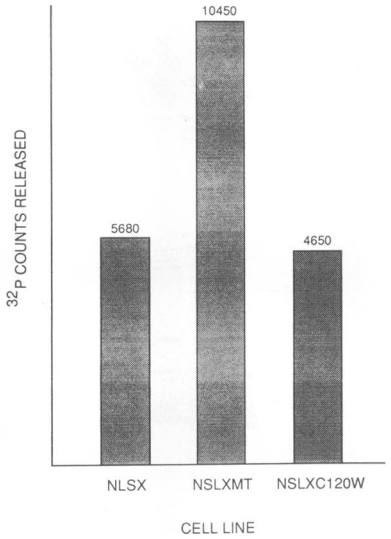

FIG. 3. MT immunoprecipitates, but not those of MTC120W, possess an associated phosphatase activity. NP-40 lysates of each cell line were immunoprecipitated with polyomavirus antitumor serum and incubated at 30°C for 15 min with casein labeled with [γ-^{32}P]ATP by using cAMP-dependent protein kinase. Samples were precipitated with 10% trichloroacetic acid, and supernatants were counted in a liquid scintillation counter. The counts (cpm) released for each cell line are indicated.

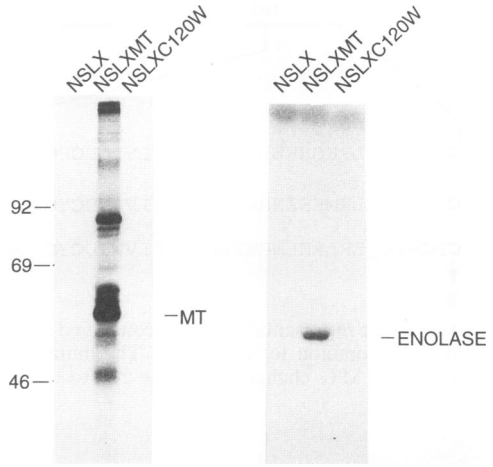

FIG. 4. MT-associated kinase activity. NP-40 lysates of the three cell lines were immunoprecipitated with polyomavirus antitumor serum. Immunoprecipitates were incubated with [γ-^{32}P]ATP at 30°C for 15 min in either the absence (left three lanes) or presence (right three lanes) of enolase as an exogenous substrate. The autoradiogram of an SDS-polyacrylamide gel is shown. The positions of MT and enolase are indicated.

resuspended in 50 μl of phosphatase buffer containing 50,000 cpm of ^{32}P-labeled casein as a substrate (see Materials and Methods). Assays were carried out on extracts from 2×10^5 to 3×10^5 cells for 15 min at 30°C. These conditions were in the linear range for both extract concentration and time (27). Associated phosphatase activity was determined by the release of ^{32}P into the supernatant after trichloroacetic acid precipitation of the reactions; the values presented were corrected for counts released by phosphatase buffer alone when incubated with the substrate under identical conditions (generally 5 to 10% of the smaller numbers). As shown in Fig. 3, the control cell line NSLX released 5,680 cpm of ^{32}P into the supernatant, whereas the wild-type MT-expressing cell line, NSLXMT, released 10,450 cpm of ^{32}P into the supernatant, indicating the presence of a phosphoseryl protein phosphatase coimmunoprecipitating with MT. The cell line NSLXMTC120W, which expresses the mutant protein MTC120W, released 4,650 cpm of ^{32}P into the supernatant. This value was at or below that obtained for the negative control cell line, indicating the absence of a protein phosphatase activity associated with the mutant protein. Thus, wild-type MT possesses an associated protein phosphatase activity, whereas the mutant, MTC120W, does not.

PP2A activity is stimulated by the addition of 1 mM $MnCl_2$ to the reaction (64) and strongly inhibited in the presence of okadaic acid, which has a 50% inhibitory dose (ID_{50}) of 1 nM (3). The protein phosphatase activity associated with MT was stimulated two- to fourfold by the inclusion of 1 mM $MnCl_2$ in the reaction, as was that found in the negative control cell line, NSLX (27). The phosphatase activity coimmunoprecipitated from both NSLX and NSLXMT cells was inhibited by okadaic acid with an ID_{50} of 1 nM (27).

MTC120W does not possess and associated kinase activity. MT associates with and activates the kinase activity of pp60$^{c\text{-}src}$, c-*fyn*, and c-*yes* (5, 8, 12, 14, 32, 38, 39). Therefore, we tested whether the mutant protein which fails to associate with PP2A could associate with pp60$^{c\text{-}src}$. We examined the various cell lines for MT-associated kinase activity. NP-40 lysates of the cell lines were immunoprecipitated with polyomavirus antitumor serum and assayed for associated kinase activity in vitro by using [γ-^{32}P]ATP. Reaction products were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. As seen in Fig. 4, wild-type MT in the NSLXMT cells coimmunoprecipitated an associated kinase activity; no kinase activity was detected in the control cells, NSLX. Interestingly, the MTC120W protein failed to coimmunoprecipitate an associated kinase activity (Fig. 4). This experiment was carried out with only [γ-^{32}P]ATP (0.135 μM), well below the K_m of the enzyme. Qualitatively similar results were obtained when 20 μM nonradioactive ATP was included in the reaction (27). We also assayed the kinase activities associated with the wild-type and mutant proteins by using enolase as an exogenous substrate. As expected, the control cells, NSLX, did not contain an associated kinase activity, whereas the MT-expressing cells did (Fig. 4). The cell line which expresses the mutant protein, MTC120W, did not possess an associated kinase activity when enolase was used as the substrate (Fig. 4). The gels shown were exposed for 3 to 4 h. Overnight (20- to 24-h) exposures did not reveal any measurable kinase activity associated with the mutant MTC120W protein (27). The NSLXMTC120W cells expressed a higher level of MT protein than did the NSLXMT cells as determined by immunoprecipitation from ^{35}S-labeled cell lysates (27). The lack of associated kinase activity is most probably due to the failure of the C120W protein to interact with pp60$^{c\text{-}src}$ since immunoprecipitation with either antitumor serum or an anti-*src* monoclonal antibody followed by immunoblotting with the other antibody failed to detect an association (27).

MTC120W fails to activate PI-3 kinase. Since MT also associates with and activates PI-3 kinase, we determined whether MTC120W was also defective in this interaction. Cell lysates were prepared as described previously (61), immunoprecipitated with antitumor serum, and incubated

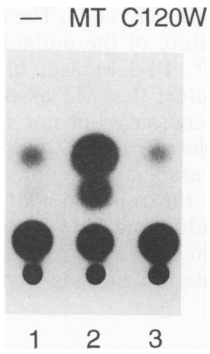

FIG. 5. MT activates expression from the c-*fos* promoter. Plates (6 cm) of BALB/c 3T3 cells were seeded at 2×10^5 cells per plate. Cells were cotransfected with FC-2 (c-*fos* promoter driving CAT expression) and either pRSV (lane 1), pRSVMT (lane 2), or pRSVC120W (lane 3), as described in Materials and Methods. Cell lysates were prepared and CAT assays were performed by standard techniques. An autoradiogram of the thin-layer chromatography plate is shown.

with phophatidylinositol and [γ-^{32}P]ATP. Lipids were extracted and separated by thin-layer chromatography. The plate was scanned and quantitated by using a Molecular Dynamics Phosphorimager. The control cells, NSLX, coprecipitated an activity which incorporated 98.5 counts into phosphatidylinositol diphosphate, whereas the MT-expressing cells coprecipitated an activity incorporating 4,846 counts. The control immunoprecipitates have 2% the associated PI-3 kinase activity as the wild-type MT immunoprecipitates. MTC120W had an associated activity which incorporated 96 counts into DPI, 2% of the level of PI-3 kinase associated with wild-type MT. Thus, the mutant MTC120W protein does not possess an associated PI-3 kinase activity above background levels. These numbers are the average of values from two experiments.

MTC120W fails to activate expression from the c-*fos* promoter. Infection of quiescent BALB/c 3T3 cells by polyomavirus results in the induction of expression of several early response genes (28, 66). Polyomavirus MT can activate the c-*fos* and c-*jun* promoters in transient-expression assays (27, 55). The ability of MT mutants to activate the c-*jun* promoter correlates with their ability to cause cell transformation (55). We tested whether the C120W mutant could activate the c-*fos* promoter. We carried out transient-transfection assays by using FC-2, a plasmid encoding the CAT gene driven by the murine c-*fos* promoter, to determine whether the mutant could activate expression from this promoter in transfected cells. When cells were cotransfected with FC-2 and pRSV, the expression vector not encoding MT, a low level of CAT activity was obtained (Fig. 5, lane 1). However, when cells were cotransfected with FC-2 and pRSVMT, an expression vector encoding wild-type MT, a 10-fold stimulation in the level of CAT activity was observed, indicating that MT could activate expression from the c-*fos* promoter (Fig. 5, lane 2). In contrast, when FC-2 was cotransfected with pRSVMTC120W, a low level of CAT activity was obtained, similar to that in control cells (Fig. 5, lane 3; compare with lane 1). Thus, the mutant protein failed to activate the c-*fos* promoter.

MTC120W fails to transform NIH 3T3 cells. We also tested the ability of the mutant protein to induce focus formation on NIH 3T3 cells. The ecotropic packaging cell line ψ-2 was transfected with retrovirus vectors encoding either wild-type or mutant MT. Supernatants from the transfected cells were used to infect NIH 3T3 cells, half of which were selected for G418 resistance to determine the infectious titer of the various stocks. The other half were allowed to grow in DME containing 5% calf serum and scored for the formation of foci overgrowing the monolayer of quiescent cells. Three plates of cells were assayed at each dilution of virus, and the number of foci were counted. As seen in Table 1, the vector alone produced a (virus) titer of 1.12×10^4 G418-resistant CFU/ml, but no foci were obtained. The MT expression vector produced a titer of 7.4×10^2 G418-resistant CFU/ml and a titer of 2.7×10^2 FFU/ml. The MTC120W expression vector gave a titer of 1.14×10^3 G418-resistant CFU/ml, but, in contrast to wild type MT, no foci were obtained. Both the wild-type MT and MTC120W proteins were expressed in a minority of individual G418-resistant colonies (2 of 9 for MT, 1 of 11 for MTC120W [27]).

TABLE 1. MT-induced focus assay on NIH 3T3 cells in three plates

Virus and concn	No. of G418^r colonies	No. of colonies/ml	No. of foci	No. of foci/ml
Neo				
10^{-2}	120, 103	1.12×10^4	0, 0, 0	0
10^{-1}	TMTC[a]		0, 0, 0	
Undil[b]	TMTC		0, 0, 0	
MT				
10^{-2}	6, 7	7.4×10^2	3, 2, 4	2.7×10^2
10^{-1}	81, 72		22, 36, 31	
Undil	TMTC		171, 223, 205	
MTC120W				
10^{-2}	12, 13	1.14×10^3	0, 0, 0	0
10^{-1}	95, 101		0, 0, 0	
Undil	TMTC			

[a] TMTC, too many to count.
[b] Undil, undiluted.

DISCUSSION

Polyomavirus MT contains a cluster of cysteine residues, Cys-X-Cys-X-X-Cys-X_{22}-Cys-X-Cys-X-X-Cys (amino acids 120 to 153), which is also present in st antigens of polyomavirus, SV40, and BK virus (25, 57). Mutation of cysteine 120 to tryptophan abolishes the ability of MT to associate with PP2A, pp60$^{c\text{-}src}$, and PI-3 kinase and renders the mutant MT defective for activation of c-*fos* expression and transformation. The analogous mutation in SV40 st was shown to abrogate its ability to cooperate with LT to transform BALB/c 3T3 cells (4).

It has been suggested that MT may interact with PP2A through the N-terminal region common to all three T antigens, because an antibody which recognizes an epitope in the common region does not precipitate the complex (46, 48). However, it seems unlikely that the PP2A-binding site would be contained solely in this region, since LT does not interact with PP2A. The results presented here indicate that the cysteine residue that is present at position 120 in MT and st but missing in LT is important for complex formation. Therefore two separate regions, the common N-terminal region and the cysteine cluster region, may be involved in complex formation. The binding domain for Rb on E1A and perhaps on SV40 LT and human papillomavirus E7 consists of two distinct regions separated by spacer regions (22, 63).

The wild-type and MTC120W mutant proteins are expressed at similar levels. By contrast, mutation of the

cysteine residues in SV40 st renders the protein unstable (29). The cysteine residues of SV40 st are thought to coordinate a zinc ion (29), possibly forming a structure which helps stabilize SV40 st. Perhaps the cysteine clusters of MT provide a structural feature which allows MT to bind PP2A and pp60$^{c\text{-}src}$. Adenovirus E1A binds to the TATA-binding protein (41). The zinc finger structure produced by four cysteine residues coordinating a zinc ion appears to be required for binding (50).

Purified SV40 st binds to the AC form of PP2A in vitro and renders its enzymatic activity similar to that of the ABC form (64), suggesting that SV40 st may act like a B subunit of PP2A. The deduced amino acid sequence of the 55-kDa B subunit of PP2A does not contain clusters of cysteine residues and does not appear to have obvious homology to SV40 st or polyomavirus st or MT (48). A cellular protein which binds Rb has a region similar to the Rb-binding region on LT, E1A, and E7 (17, 35), suggesting that DNA tumor viruses use binding domains found on cellular proteins. It would be of interest to determine the AC-binding domain on the B subunit and look for a similar region in the T antigens which bind PP2A.

MT-PP2A complexes isolated from cells have phosphatase activity in vitro; thus, there is not a complete inhibition of phosphatase activity as a result of binding by MT. SV40 st can apparently inhibit (e.g., towards myosin light chain, myelin basic protein, SV40 LT, or p53) or enhance (e.g., toward histone H1) the activity of PP2A (54, 64). It will be important to determine the effect of MT on PP2A activity by using different substrates. We are looking for substrates with altered serine or threonine phosphorylation resulting from alterations in PP2A activity by MT.

The C120W mutant protein fails to associate with pp60$^{c\text{-}src}$ and lacks an associated kinase activity. Markland and Smith observed that mutation of any of the cysteine residues to serines abolished the ability of MT to activate pp60$^{c\text{-}src}$ kinase activity (42). The C120W mutation might affect the association of MT with pp60$^{c\text{-}src}$ directly by altering the structure of MT or indirectly by affecting PP2A binding to MT. Most of MT is found in association with PP2A (31), suggesting that failure to associate with PP2A could be a primary defect. For example, PP2A might alter the phosphorylation state of MT, and this, in turn, might influence its binding to pp60$^{c\text{-}src}$. It is also possible that binding to pp60$^{c\text{-}src}$ involves determinants on both MT and PP2A.

Phosphorylation of MT on tyrosine 315, presumably by activated pp60$^{c\text{-}src}$ in the complex, is important for the association of MT with PI-3 kinase, most probably through association with the SH2 domain of the p85 subunit (13, 36, 44). The MTC120W protein fails to activate PI-3 kinase, consistent with the lack of protein kinase activity associated with this mutant.

Two other mutations in MT, SD15 and NG59, affect the association of MT with both PP2A and pp60$^{c\text{-}src}$ (reference 42 and references therein). The SD15 protein carries a large internal deletion (amino acids 83 to 129) which includes the first cluster of cysteine residues. The NG59 protein contains an isoleucine inserted between amino acids 178 and 179, downstream from the cysteine clusters, which may indicate a third region important for PP2A and pp60$^{c\text{-}src}$ association.

The MTC120W mutant fails to activate the c-*fos* promoter. Wild-type MT activates the c-*fos* promoter through multiple response elements in transient transfections (27, 55). A signal sent by any of these molecules could induce c-*fos* expression.

MTC120W is defective for transformation as measured by focus formation on NIH 3T3 cells. The transformation defect could result from failure of the mutant protein to associate with PP2A, pp60$^{c\text{-}src}$, PI-3 kinase, or combinations of all three proteins. It is clear that MT association with pp60$^{c\text{-}src}$ and PI-3 kinase is necessary but not sufficient for transformation (21). The role of MT-PP2A complexes is less well defined. MT-PP2A association could promote binding to pp60$^{c\text{-}src}$. However, the remainder of the MT-PP2A complexes may play an additional role in transformation. We are making mutations in other regions of MT as a genetic approach to dissociate MT binding to pp60$^{c\text{-}src}$ from binding to PP2A.

ACKNOWLEDGMENTS

This publication was supported by grants CA13884 and CA14195 from the National Cancer Institute.

The MT monoclonal antibody PAb815 was a gift from Joe Bolen, Bristol-Myers Squibb, Princeton, N.J.

REFERENCES

1. **Asselin, C., and M. Bastin.** 1985. Sequences from polyomavirus and simian virus 40 large T genes capable of immortalizing primary rat embryo fibroblasts. J. Virol. **56**:958–968.
2. **Berger, H., and E. Wintersberger.** 1986. Polyomavirus small T antigen enhances replication of viral genomes in 3T6 mouse fibroblasts. J. Virol. **60**:768–770.
3. **Bialojan, C., and A. Takai.** 1988. Inhibitory effect of a marine sponge toxin, okadaic acid, on protein phosphatases. Biochem. J. **256**:283–290.
4. **Bikel, I., H. Mamon, E. L. Brown, J. Boltax, M. Agha, and D. L. Livingston.** 1986. The t-unique coding domain is important to the transformation maintenance function of the simian virus 40 small t antigen. Mol. Cell. Biol. **6**:1172–1178.
5. **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.
6. **Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan.** 1987. Cell transformation by pp60$^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
7. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**:2745–2752.
8. **Cheng, S. H., R. Harvey, P. Espino, K. Semba, T. Yamamoto, K. Toyoshima, and A. E. Smith.** 1988. Peptide antibodies to the human c-*fyn* gene product demonstrate pp59$^{c\text{-}fyn}$ is capable of complex formation with the middle-T antigen of polyomavirus. EMBO J. **7**:3845–3855.
9. **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.
10. **Cohen, P.** 1989. The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58**:453–508.
11. **Cooper, J. A., F. S. Esch, S. S. Taylor, and T. Hunter.** 1984. Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases *in vivo* and *in vitro*. J. Biol. Chem. **259**:7835–7841.
12. **Courtneidge, S. A.** 1985. Activation of the pp60$^{c\text{-}src}$ kinase by middle T antigen binding or by dephosphorylation. EMBO J. **4**:1471–1477.
13. **Courtneidge, S. A., and A. Heber.** 1987. An 81 kd protein complexed with middle T antigen and pp60$^{c\text{-}src}$: a possible phosphatidylinositol kinase. Cell **50**:1031–1037.
14. **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.
15. **Cowie, A., J. de Villiers, and R. Kamen.** 1986. Immortalization of rat embryo fibroblasts by mutant polyomavirus large T antigens deficient in DNA binding. Mol. Cell. Biol. **6**:4344–4352.
16. **DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M.**

Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell **54**:275–283.

17. **Defeo-Jones, D., P. S. Huang, R. E. Jones, K. M. Haskell, G. A. Vuocolo, M. G. Hanobik, H. E. Huber, and A. Oliff.** 1991. Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. Nature (London) **352**:251–254.
18. **Deschamps, J., F. Meijlink, and I. M. Verma.** 1985. Identification of a transcriptional enhancer element upstream from the proto-oncogene *fos*. Science **230**:1174–1177.
19. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**:725–737.
20. **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.
21. **Druker, B. J., L. E. Ling, B. Cohen, T. M. Roberts, and B. S. Schaffhausen.** 1990. A completely transformation-defective point mutant of polyomavirus middle T antigen which retains full associated phosphatidylinositol kinase activity. J. Virol. **64**:4454–4461.
22. **Dyson, N., R. Bernards, S. H. Friend, L. R. Gooding, J. A. Hassell, E. O. Major, J. M. Pipas, T. Vandyke, and E. Harlow.** 1990. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. J. Virol. **64**:1353–1356.
23. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–936.
24. **Escobedo, J. A., S. Navankasattusas, W. M. Kavanaugh, D. Milfay, V. A. Fried, and L. T. Williams.** 1991. cDNA cloning of a novel 85 kD protein that has SH2 domains and regulates binding of PI3-kinase to the PDGF β receptor. Cell **65**:75–82.
25. **Friedmann, T., R. F. Doolittle, and G. Walter.** 1978. Amino acid sequence homology between polyoma and SV40 tumour antigens deduced from nucleotide sequences. Nature (London) **274**:291–293.
26. **Garcea, R. L., D. A. Talmage, A. Harmatz, R. Freund, and T. L. Benjamin.** 1989. Separation of host range from transformation functions of the hr-t gene of polyomavirus. Virology **168**:312–319.
27. **Glenn, G., and W. Eckhart.** Unpublished data.
28. **Glenn, G., and W. Eckhart.** 1990. Transcriptional regulation of early-response genes during polyomavirus infection. J. Virol. **64**:2193–2201.
29. **Goswami, R., B. Turk, K. Enderle, A. Howe, and K. Rundell.** 1992. Effect of zinc ions on the biochemical behavior of simian virus 40 small-t antigen expressed in bacteria. J. Virol. **66**:1746–1751.
30. **Grussenmeyer, T., A. Carbone-Wiley, K. H. Scheidtmann, and G. Walter.** 1987. Interaction between polyomavirus medium T antigen and three cellular proteins of 88, 61, and 37 kilodaltons. J. Virol. **61**:3902–3909.
31. **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.
32. **Horak, I. D., T. Kawakami, F. Gregory, K. C. Robbins, and J. B. Bolen.** 1989. Association of $p60^{fyn}$ with middle tumor antigen in murine polyomavirus-transformed rat cells. J. Virol. **63**:2343–2347.
33. **Ito, Y., T. Noda, M. Satake, T. Robins, and M. Gonzatti-Haces.** 1986. Growth-stimulating activity of polyomavirus small T antigen. Cancer Cells **4**:413–418.
34. **Jat, P. S., and P. A. Sharp.** 1986. Large T antigens of simian virus 40 and polyomavirus efficiently establish primary fibroblasts. J. Virol. **59**:746–750.
35. **Kaelin, W. G., Jr., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70**:351–364.
36. **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: an 85 kD phosphoprotein and phosphatidylinositol kinase activity. Cell **50**:1021–1029.
37. **Kaplan, P. L., S. Simon, C. A. Cartwright, and W. Eckhart.** 1987. cDNA cloning with a retrovirus expression vector: generation of a $pp60^{c\text{-}src}$ cDNA clone. J. Virol. **61**:1731–1734.
38. **Kornbluth, S., M. Sudol, and H. Hanafusa.** 1987. Association of the polyomavirus middle-T antigen with c-*yes* protein. Nature (London) **325**:171–173.
39. **Kypta, R. M., A. Hemming, and S. A. Courtneidge.** 1988. Identification and characterization of $p59^{fyn}$ (a *src*-like protein tyrosine kinase) in normal and polyoma virus transformed cells. EMBO J. **7**:3837–3844.
40. **Larose, A., N. Dyson, M. Sullivan, E. Harlow, and M. Bastin.** 1991. Polyomavirus large T mutants affected in retinoblastoma protein binding are defective in immortalization. J. Virol. **65**:2308–2313.
41. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk.** 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67**:365–376.
42. **Markland, W., and A. E. Smith.** 1987. Mutants of polyomavirus middle-T antigen. Biochim. Biophys. Acta **907**:299–321.
43. **Martens, I., S. A. Nilsson, S. Linder, and G. Magnusson.** 1989. Mutational analysis of polyomavirus small-T-antigen functions in productive infection and in transformation. J. Virol. **63**:2126–2133.
44. **Otsu, M., I. Hiles, I. Gout, M. J. Fry, F. Ruiz-Larrea, G. Panayotou, A. Thompson, R. Dhand, J. Hsuan, N. Totty, A. D. Smith, S. J. Morgan, S. A. Courtneidge, P. J. Parker, and M. D. Waterfield.** 1991. Characterization of two 85 kD proteins that associate with receptor tyrosine kinases, middle T/$pp60^{c\text{-}src}$ complexes, and PI3-kinase. Cell **65**:91–104.
45. **Pallas, D. C., V. Cherington, W. Morgan, J. DeAnda, D. Kaplan, B. Schaffhausen, and T. M. Roberts.** 1988. Cellular proteins that associate with the middle and small T antigens of polyomavirus. J. Virol. **62**:3934–3940.
46. **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.
47. **Pallas, D. C., L. K. Shahrik, B. L. Martin, S. Jaspers, T. B. Miller, D. L. Brautigan, and T. M. Roberts.** 1990. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell **60**:167–176.
48. **Pallas, D. C., W. Weller, S. Jaspers, T. B. Miller, W. S. Lane, and T. M. Roberts.** 1992. The third subunit of protein phosphatase 2A (PP2A), a 55-kilodalton protein which is apparently substituted for by T antigens in complexes with the 36- and 63-kilodalton PP2A subunits, bears little resemblance to T antigens. J. Virol. **66**:886–893.
49. **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" roden fibroblast cell lines. Proc. Natl. Acad. Sci. USA **80**:4354–4358.
50. **Ricciardi, R. P.** Personal communication.
51. **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.
52. **Schaffhausen, B., J. Silver, and T. L. Benjamin.** 1978. Tumor antigen(s) in cells productively infected by wild-type polyoma virus and mutant NG18. Proc. Natl. Acad. Sci. USA **75**:79–83.
53. **Scharfmann, R., J. H. Axelrod, and I. M. Verma.** 1991. Long-term *in vivo* expression of retrovirus-mediated gene transfer in mouse fibroblast implants. Proc. Natl. Acad. Sci. USA **88**:4626–4630.
54. **Scheidtmann, K. H., M. C. Mumby, K. Rundell, and G. Walter.** 1991. Dephosphorylation of simian virus 40 large-T antigen and p53 protein by protein phosphatase 2A: inhibition by small-t antigen. Mol. Cell. Biol. **11**:1996–2003.

55. **Schönthal, A., S. Srinivas, and W. Eckhart.** 1992. Induction of c-*jun* protooncogene expression and transcription factor AP-1 activity by the polyoma virus middle-sized tumor antigen. Proc. Natl. Acad. Sci. USA **89:**4972–4976.
56. **Templeton, D., S. Simon, and W. Eckhart.** 1986. Truncated forms of the polyomavirus middle T antigen can substitute for the small T antigen in lytic infection. J. Virol. **57:**367–370.
57. **Tooze, J.** 1981. Molecular biology of tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
58. **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.
59. **Walter, G., A. Carbone-Wiley, B. Joshi, and K. Rundell.** 1988. Homologous cellular proteins associated with simian virus 40 small t antigen and polyomavirus medium T antigen. J. Virol. **62:**4760–4762.
60. **Walter, G., R. Ruediger, C. Slaughter, and M. Mumby.** 1990. Association of protein phosphatase 2A with polyoma virus medium tumor antigen. Proc. Natl. Acad. Sci. USA **87:**2521–2525.
61. **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.
62. **Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg, and E. Harlow.** 1988. Association between an oncogene and an antioncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature (London) **334:**124–129.
63. **Whyte, P., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell **56:**67–75.
64. **Yang, S.-I., R. L. Lickteig, R. Estes, K. Rundell, G. Walter, and M. C. Mumby.** 1991. Control of protein phosphatase 2A by simian virus 40 small-t antigen. Mol. Cell. Biol. **11:**1988–1995.
65. **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.
66. **Zullo, J., C. D. Stiles, and R. L. Garcea.** 1987. Regulation of c-*myc* and c-*fos* mRNA levels by polyomavirus: distinct roles for the capsid protein VP_1 and the viral early proteins. Proc. Natl. Acad. Sci. USA **84:**1210–1214.