JOURNAL OF VIROLOGY, Feb. 1987, p. 285–292
0022-538X/87/020285-08$02.00/0

Vol. 61, No. 2

Mapping of the Amino-Terminal Half of Polyomavirus Middle-T Antigen Indicates that This Region Is the Binding Domain for pp60$^{c\text{-}src}$

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Received 22 August 1986/Accepted 15 October 1986

The majority of the carboxy-terminal half of polyomavirus middle-T antigen has been variously mutated and, with the exception of the putative membrane-binding domain (amino acids 394 to 415), was found to be largely dispensible for the transforming activity of the protein. A comparison of the small-T antigen amino acid sequences (equivalent to the region of middle-T encoded by exon 1) of simian virus 40, BK virus, polyomavirus, and a recently described hamster papovavirus highlighted regions of potential interest in mapping functions to the amino-terminal half of polyomavirus middle-T antigen. The regions of interest include amino acids 168 to 191 (previously investigated by this group [S. H. Cheng, W. Markland, A. F. Markham, and A. E. Smith, EMBO J. 5:325–334, 1986]), two cysteine-rich clusters (amino acids 120 to 125 and 148 to 153), and amino acids 92 to 117 (within the limits of the previously described *hr-t* mutant, SD15). Point mutations, multiple point mutations, and deletions were made by site-specific and site-directed mutagenesis within the cysteine-rich clusters and residues 92 to 117. Studies of the transforming ability of the altered middle-T species demonstrated that this activity is highly sensitive to amino acid changes. All four regions (as defined above) within the amino-terminal half of middle-T have now been studied in detail. The phenotype of the mutants is predominantly transformation defective, and the corresponding variant middle-T species are characterized by being either totally or severely handicapped in the ability to associate actively with pp60$^{c\text{-}src}$. Whether the mutations affect the regions of interaction between middle-T and pp60$^{c\text{-}src}$ or simply interfere with the overall conformation of this domain is not known. However, there would appear to be a conformational constraint on this portion of the molecule with regard to its interaction with pp60$^{c\text{-}src}$ and by extension to the ability of the middle-T species to transform.

The early region of the polyomavirus genome encodes three proteins detected as tumor (T) antigens, large-T, middle-T, and small-T (26), which share common regions of amino acid sequence. Large-T, middle-T, and small-T have a common amino terminus of 79 amino acids, while middle-T and small-T share a further stretch of amino acids up to and including residue 191, this boundary being determined by the common donor splice site for the two molecules. In essence, polyomavirus small-T is the region of middle-T encoded by exon 1 plus four extra amino acids at its carboxy terminus. The carboxy-terminal half of middle-T is encoded by exon 2 of the middle-T gene (which is shared with large-T) and is composed of a unique sequence of amino acids from residues 192 to 421 (23) derived from an alternative reading frame.

Middle-T when expressed separately from the other polyomavirus tumor antigens has the ability to transform the growth properties of established rodent cells (27). A current model to explain the biochemical basis of the action of middle-T (reviewed by S. H. Cheng, W. Markland, and A. E. Smith, *in* P. Kahn and T. Graf, ed., *Oncogenes and Growth Control* in press) centers upon the association of middle-T with pp60$^{c\text{-}src}$ (6). This association has been shown to enhance the tyrosyl kinase activity of the proto-oncogene product (2, 5) and alter the degree of phosphorylation of middle-T in vitro (8, 22, 24) and of pp60$^{c\text{-}src}$ in vitro (29) and in vivo (5). It has been proposed (5) that the associated middle-T prevents the phosphorylation of tyrosine residues at the carboxy terminus of pp60$^{c\text{-}src}$ and that this masking event induces the activation of the complexed pp60$^{c\text{-}src}$. The activation of pp60$^{c\text{-}src}$ or possibly an alteration in the substrate specificity of this kinase is thought to trigger the transformation process. A further correlation has been shown to exist between the presence of various transforming and nontransforming mutants of middle-T in cell lysates and phosphatidylinositol kinase activity (13, 28). Protein kinase C activity has also been found to be associated with middle-T (17, 20).

A large number of polyomavirus early-region mutants have been generated, and these fall into one of three groups. The *ts* mutants contain lesions within the unique region of the large-T antigen (23), the *hr-t* mutants (reviewed in reference 1) contain lesions which affect both the middle-T and small-T antigens, while a third group of mutants, the *ml-t* mutants, map to the region of the genome encoding both middle-T and large-T (11). Many workers have made mutations in the region of the polyomavirus genome encoding the unique region of middle-T expecting to demonstrate that this part of the molecule is responsible for its transforming properties. Surprisingly, the results indicate that apart from the hydrophobic domain (discussed in reference 15), no contiguous sequence of amino acids within the carboxy-terminal half of the molecule is absolutely required for middle-T-induced transformation. Comparatively few mutations have been reported that map to the amino-terminal half of middle-T. Of these, the original *hr-t* mutants were uniformally defective in transformation (3). Amino-terminal truncations also have a severe effect on the transforming capability of the encoded middle-T species (14, 25), and these in turn fail to display an associated kinase activity. More recently, smaller amino-terminal deletion mutants and

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one in which the penultimate amino-terminal amino acid aspartic acid (residue 2) was replaced by alanine and tryptophan displayed a phenotype identical to that of the amino-terminal truncations (D. Cook, personal communication).

In an attempt to identify regions within the amino-terminal half of middle-T of potential importance in the transformation process, we carried out a computer analysis of the tumor antigens of polyomavirus, simian virus 40 (SV40), BK virus, and the recently described hamster papovavirus. This highlighted several regions of interest. The results reported here demonstrate that the transforming activity and $pp60^{c\text{-}src}$ association of the altered middle-T species are highly sensitive to amino acid changes at several other sites within the amino-terminal half of the molecule.

MATERIALS AND METHODS

Cells and assays. Purified plasmids were introduced into Rat-1 cells by the calcium phosphate transfection method (10). Middle-T-transformed cells were selected as dense foci of cells overgrowing the cell monolayer. The neomycin resistance gene (pSV2neo) was similarly cotransfected with the plasmids in a ratio of 10:1, and neomycin-resistant cellular clones were selected by growth in the presence of the antibiotic G418. At least 50% of the neomycin-resistant cell lines analyzed also expressed middle-T. The cell culture growth conditions, preparation of cell lysates, immunoprecipitations, and in vitro kinase assays used in this study have all been described previously (5, 6, 24). The use and comparison of in vitro qualitative and quantitative kinase assays are as detailed in reference 16.

Computer analysis. A comparison of the amino acid sequences of the papovavirus T antigens was carried out with the Beckman Microgenie sequence analysis program. The program compares the amino acid sequences of proteins by four different methods: homology comparison, matrix comparison, alignment of protein sequences, and protein alignment by similarity.

In vitro mutagenesis. (i) Polyomavirus middle-T cysteine region deletions mutants. The 1,077-base-pair *EcoR*I-*Pst*I fragment of the polyomavirus genome was cloned into the single-stranded bacteriophage M13mp8. This construct has previously been used to introduce mutations in sequences encoding polyomavirus middle-T (16). Mutagenesis was carried out to delete individually the DNA sequences encoding the cysteine regions utilizing the synthetic oligonucleotides 5′-CTT · AAA · TAC · AGC · TCA · CTG · GTT · AGA · AAG · CAA-3′ (30mer) and 5′-CTA · GTA · CTT · GGA · GAA · TAC · ATG · CAA · TGG · TTT-3′ (30mer) to remove the first and second cysteine clusters, respectively. The required deletions were screened for by radiolabeled probe hybridization with 18mer synthetic oligonucleotides representing the six amino acid codons deleted from each cysteine-encoded region.

Selected phage were grown in the bacterial strain JM101, from which the double-stranded replicative-form DNA containing the deletions was extracted and digested with *EcoR*I and *Pst*I, followed by religation of the early-region fragment into the parental pAS101 plasmid. The mutant plasmids in which nucleotides 530 to 547 (amino acids 120 to 125) and nucleotides 614 to 631 (amino acids 148 to 153) had been deleted were named pDC1 and pDC2, respectively (deletion of cysteine region 1 and cysteine region 2). The appropriate deletions were demonstrated by restriction enzyme analysis of the mutant plasmids (*Pst*I-*Sac*I for pDC1 and *Sac*I-*Ava*I for pDC2) followed by analysis of the DNA fragments by 4% Nusieve gel electrophoresis. The deletions were confirmed by DNA sequencing by the dideoxynucleotide method.

(ii) Bisulfite mutagenesis of DNA encoding the cysteine regions of polyomavirus middle-T. The cysteine codons (TGT and TGC) are susceptible to mutagenesis with sodium bisulfite (C-to-T and the complementary G-to-A transitions), generating tyrosine codons in their place. For each cysteine region a looped heteroduplex was created (as detailed in reference 12) by using wild-type plasmid and either pDC1 or pDC2 which generated single-stranded DNA at the sites encoding the first and second cysteine region clusters, respectively. Bisulfite mutagenesis was carried by the methods previously described (12). Mutants were screened by radiolabeled probe hybridization with a synthetic oligonucleotide of wild-type sequence for each cysteine region. The mutant plasmids were sequenced and named the pBC1 and the pBC2 series of mutants (bisulfite mutants of the cysteine regions).

(iii) Polyomavirus middle-T SD15 region deletion mutants. The lesion in the original SD15 mutant (3), nucleotides 419 to 562, contains a *Pst*I site at its center, and this was used as the starting position to make deletions. The wild-type plasmid contains two *Pst*I sites at nucleotides 4749 (within the ampicillin resistance gene) and 484. The plasmid was partially digested with *Pst*I followed by the extraction and purification of the linearized molecule. The linearized plasmid contained a 3′ overhang after *Pst*I digestion which was made flush with T4 DNA polymerase in the presence of deoxynucleotides. A synthetic *Bgl*II linker (5′-CAGATCTG-3′) was blunt-end ligated onto the flush ends, followed by *Bgl*II digestion and recircularization of the plasmid. The plasmids were transfected into *Escherichia coli* ED8767 and selected for by the loss of a *Pst*I site and the inclusion of a unique *Bgl*II site.

Deletions were made in the plasmid utilizing this new unique restriction enzyme site as a starting point. The plasmid was linearized with *Bgl*II followed by digestion with *Bal* 31 (a double-stranded exonuclease) for various lengths of time, as detailed by Markland et al. (15). *Bgl*II linkers were reintroduced followed by purification of the recircularized plasmids and transfection into ED8767. A series of deletion mutants were generated spanning and extending from the *Pst*I site of the parent plasmid. The deleted plasmids were sequenced to determine the extent of the lesion and the phase of the middle-T-encoding region of the plasmid. They were designated the pPB series of mutants (*Pst-Bgl*II mutants).

(iv) Bisulfite mutagenesis of DNA encoding amino acids 102 to 110 of polyomavirus middle-T antigen. A gapped heteroduplex was made with wild-type plasmid and pPB3 by a method similar to that described in reference 16. This generated a single-stranded region of DNA which was mutated by the sodium bisulfite method described above. Mutants were selected for by the loss of the *Pst*I site at nucleotide 1078 followed by DNA sequence analysis. These mutants were designated the pBP series of mutants (bisulfite mutants of the *Pst*I site).

RESULTS

Amino acid sequence comparisons of tumor antigens of SV40, BK virus, polyomavirus, and hamster papovavirus. Analysis of the predicted amino acid sequences of polyomavirus and hamster papovavirus middle-T antigens highlighted regions of conserved sequence. Assuming that the putative middle-T of hamster papovavirus has the same

function as the polyomavirus equivalent, then the sequences encoded by exons 1 and 2 show an unexpected disparity. The amino-terminal half of each molecule demonstrates a high degree of homology, while the carboxy-terminal halves are surprisingly dissimilar, containing only two homologous sequences. One is the region around tyrosine-315 of polyomavirus which has two possible homologous sequences in the hamster papovavirus middle-T amino acid sequence (W. Markland, S. H. Cheng, B. L. Roberts, R. Harvey, and A. E. Smith, *in* M. Botchan, T. Grodzicker, and P. Sharp, ed., *Cancer Cells 4*, in press), and the second is the putative membrane-binding domain of the middle-T molecules (15).

The early regions of the SV40 and BK viruses encode a large-T and a small-T antigen species but, unlike polyomavirus and hamster papovavirus, no middle-T species. Comparison of the amino acid sequences of SV40, BK virus, polyomavirus, and hamster papovavirus small-T antigens (and hence of the amino-terminal halves of the appropriate middle-T antigens) suggests a division of the molecules into four sections as judged by the degree of homology and known biological function (Fig. 1). Amino acids 1 to 78 (section 1) are shared with large-T in the case of SV40 and BK virus or with large-T and middle-T in the case of polyomavirus and hamster papovavirus. This section demonstrates a high degree of homology and contains an amino acid sequence (42-His-Pro-Asp-Lys-Gly-Gly-47) which is absolutely conserved in all the T antigens.

The second section (amino acids 79 to 114) contains two sequences of particular homology between hamster papovavirus and polyomavirus at amino acids 79 to 91 and 105 to 114. Interestingly, although the amino acid sequences of the BK virus and SV40 small-T antigens are exceptionally homologous with one another throughout most of their sequence, this is not the case for the region bounded by amino acids 79 to 91. Since this region is not conserved in BK virus and SV40, it may be that it is unimportant to the function of small-T antigens. Conversely, it is potentially of importance to the middle-T molecule. This region of polyomavirus middle-T has not been previously investigated other than in the transformation-defective *hr-t* mutant SD15 which contains a large deletion in this and the adjacent section.

The two cysteine-rich regions, previously noted as being conserved between the small-T antigens of SV40 and polyomavirus and certain polypeptide hormones (9), are contained within amino acids 118 to 159 (section 3). There is a high degree of homology among all four small-T antigens within this region not only in the cysteine residues themselves but also the flanking regions.

BK virus and SV40 small-T antigens are 174 and 172 amino acids long, respectively, while hamster papovavirus small-T is 195 amino acids and polyomavirus small-T is 196 amino acids in length. The additional amino acids (168 to 191 or section 4) of polyomavirus small-T (and hence middle-T) are strongly conserved in hamster papovavirus (Fig. 1). Since the small-T molecules of BK virus and SV40 function without this sequence it is conceivable that it is not of importance to small-T. A study of part of this region in polyomavirus middle-T (4) has already shown it to be crucial to its transforming function.

To investigate the amino-terminal half of middle-T in more detail, we studied two sites, the cysteine-rich regions of section 3 and sequences within section 2.

Mutations within DNA encoding cysteine-rich regions in the amino-terminal half of middle-T. The DNA encoding the middle-T antigen of polyomavirus was mutated to create deletions in two separate plasmids, pDC1 and pDC2, in which the first and second cluster of cysteine residues were predicted to be removed (Fig. 2). Both mutant plasmids were totally defective in transforming ability when tested in a focus formation assay on a monolayer of Rat-1 cells, al-

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Section 1.          10          20          30          40
MUPY    MDRVLSRADK  ERLLELLKLP  RQLWGDPGRM  QQAYKQQSLL
HAPV    MDRILTKEEK  QALISLLDLE  PQYWGDYGRM  QKCYKKKCLQ
SV40    MDKVLNREES  LQLMDLLGLE  RSAWGNIPLM  RKAYLKKCKE
BKV     MDKVLNREES  MELMDLLGLE  RAAWGNLPLM  RKAYLRKCKE
        ** #* ###     *  ** *#  #  **    *   ##*  ##

                50          60          70     75
MUPY    LHPDKGGSHA  LMQELNSLWG  TFKTEVYNLR  MNLGG
HAPV    LHPDKGGDEE  LMQQLNTLWT  KLKDGLYRVR  LLLGP
SV40    FHPDKGGDEE  KMKKMNTLYK  KMEDGVKYAH  QPDFG
BKV     FHPDKGGDED  KMKRMNTLYK  KMEQDVKVAH  QPDFG
         *******##    *   *#*    #    #         #

Section 2.
MUPY  79  QVRRLHADGW NLSTKDTFGD RYYQRFCRMP LTCLVNVKY   117
HAPV  77  QVRRLGKDQW NLSLQQTFSG TYFRRLCRLP ITCLRNNGI  115

SV40  79  DA    TEVF A SSLNP GV D  AMYCKQP  ECAKKM     107
                                      W
BKV   78  SSSEVCAD F PLCP DTL    Y   CKEP I CSKKP     105
                                      W
                  #    ##   #     #    *  *   *

Section 3.
MUPY  119 SCSCILCLLRKQHR 132     142 CLVLGECFCLECWMQWFGT 160
HAPV  117 TCNCILCLLRKQHF 131     140 CLVLGECYCIDCFALWFGL 158
SV40  110 NCICLLCLLRMKHE 123     132 PLVWVDCYCFDCFRMWFGL 150
BKV   108 HCPCMLCQLRLRHL 121     130 PLVWIDCYCIDCFTQWFGL 148
          * * **#**  *                 **   *#* #*#  ***#

Section 4.
MUPY  168 LYADFIASMP IDWLDLDVHS VYNPRLSP  196
HAPV  167 LYAQFLAPIP VDWLDLNVHE VYNPASGP  197

SV40  152 L CE  GTL  LLWCDIIGQT TYRD LKL  174
BKV   150 L TE  ETLQ W WVQIIGET PFRD LKL  172
          *          * #       #   #
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FIG. 1. Detailed comparison of the amino acid sequences of the small-T antigens of polyomavirus (MUPV), hamster papovavirus (HAPV), SV40, and BK virus. The four sections are as defined in Fig. 3 with the numbers referring to the amino acids of each individual antigen. * indicates complete homology, while # indicates near homology (75%). The underlining in sections 2 and 4 indicates the homologies existing in the pairs of small-T antigens from either polyomavirus and hamster papovavirus or SV40 and BK virus. Abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, aspargine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

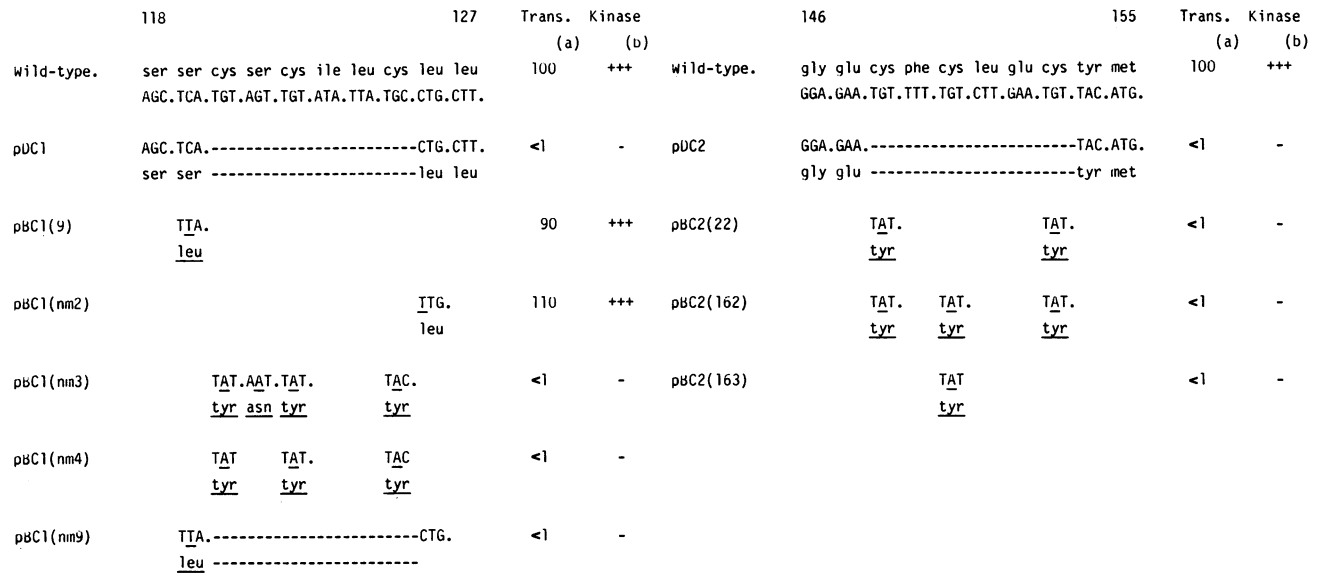

FIG. 2. Predicted lesions in the cysteine cluster regions of middle-T antigen. The amino acid sequences of the wild-type molecule are shown, and the individual lesions of the cysteine region mutants are indicated. The dotted lines indicate a deletion, and the underlined residues indicate the alterations in DNA sequence determined and the predicted amino acid changes. (a) Transforming ability of the mutants as determined by focus formation assay and relative to that of the wild-type plasmid (100%). (b) Associated kinase (phosphate acceptor) activity of the mutant-encoded middle-T species relative to wild type. +++, Wild type; −, no kinase activity.

though both were able to express an appropriate middle-T species in rodent cells (see below).

Two further sets of mutants were created, the pBC1 and pBC2 series (Fig. 2), which encoded middle-T species containing changes in the first and second cysteine clusters, respectively. pBC1(nm4) contained three G-to-A transitions causing cysteine codons to be altered to tyrosine codons at residues 120, 122, and 125. pBC1(nm3) contained the same mutations plus an extra G-to-A transition causing an alteration of serine-121 to asparagine. pBC(9) contained a point mutation causing a serine-to-leucine alteration at residue 119, while pBC1(nm2) contained a C-to-T transition which does not cause a change in the amino acid sequence. pBC1(nm9) contained both a deletion and an extra point mutation causing a serine-to-leucine alteration at residue 119. The mutants which contained predicted changes to the cysteine residues were transformation defective when tested in a focus formation assay, although all were able to express a middle-T species. Plasmids pBC1(9) and pBC1(nm2) transformed at levels comparable to that of the wild type.

Mutants with predicted alterations in the second cluster of cysteine residues were also generated (Fig. 2). pBC2(163) encoded a single cysteine-to-tyrosine change at residue 150, pBC2(22) contained two G-to-A transitions causing cysteine-to-tyrosine alterations at residues 148 and 153, and pBC2(162) encoded a middle-T species in which all three cysteine residues were converted to tyrosine residues in this cluster. Each of the pBC2 series of mutants was transformation defective when assayed by focus formation on a monolayer of Rat-1 cells, although they were able to express an appropriately sized middle-T species.

The above plasmids encode altered middle-T and small-T species together with an unmutated large-T fragment. While middle-T alone has been demonstrated to be capable of transformation of established cells, the possibility remains that an altered small-T molecule may influence the transforming ability of the middle-T species (see reference 18). To ensure that this is not the case, we recloned pDC1 and pDC2 into a vector containing the cDNA of the middle-T gene. When the middle-T species containing the cysteine region deletions were expressed in the absence of other T-antigens they remained unable to transform.

Mutations within DNA encoding amino acids of the SD15 region of middle-T. The SD15 region of DNA was mutated for two reasons. First, a computer analysis indicated that it may be of potential interest, and second, this region is removed by the lesion in the transformation-defective *hr-t* mutant SD15 (amino acids 83 to 129). pPB3 was created by cleaving wild-type plasmid at the *Pst*I site (nucleotide 484) followed by the insertion of a synthetic *Bgl*II linker. In this instance the creation of flush ends with T4 polymerase also generated a small deletion equivalent to nine amino acids which was replaced by three amino acids encoded by the linker (Fig. 3). A further series of deletion mutants were generated by *Bal* 31 digestion extending from the *Pst*I site, of which pPB1 and pPB2 are shown in Fig. 3. The boundaries of the deletions are as shown together with the inserted amino acids encoded by the *Bgl*II linker. When tested in a focus formation assay each of the deletion-linker-insertion mutants was totally defective in its ability to transform established rodent cells in vitro, although they were able to express a deleted middle-T species.

A more detailed analysis of this region was carried out by chemical mutagenesis. pBP1 contains two G-to-A transitions causing arginine (103) to be converted to glutamine and cysteine (105) to be altered to tyrosine, while pBP2 contains two conserved mutations and hence encodes a wild-type molecule. Plasmid pBP3 encodes a middle-T species with three amino acid alterations, arginines (99 and 106) to lysine residues and a seemingly fortuitous mutation (not bisulfite induced) causing threonine (110) to be changed to isoleucine. Plasmids pBP4 and pBP5 each contain three C-to-T transi-

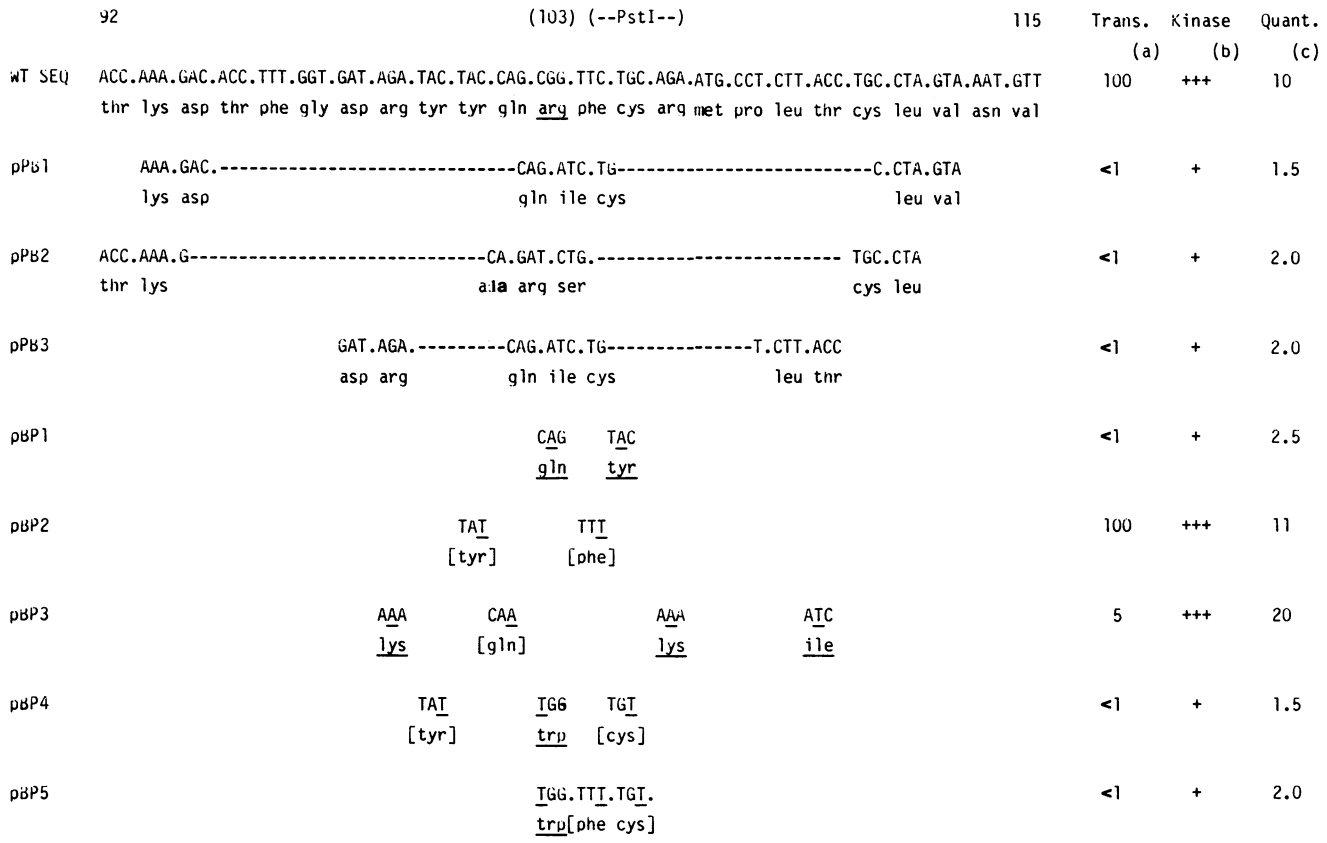

FIG. 3. Predicted lesions in the SD15 region of middle-T antigen. The wild-type amino acid sequence is shown, and the individual lesions of this region are as indicated. The dotted line indicates a deletion, with the introduced amino acids as a result of the inserted *Bgl*II linker. The underlined residues indicate the alterations in the DNA sequence and the predicted amino acid changes. Amino acids in brackets indicate conserved alterations. (a) Transforming ability of the mutants as determined by focus formation assays relative to that of wild type (100%). (b) Associated in vitro kinase (phosphate acceptor) assays determined for the mutant-encoded middle-T species relative to wild type. +++, Wild type; +, minimal levels of kinase activity. (c) Quantitative in vitro kinase (pp60$^{c\text{-}src}$ activation) assays determined for the mutant-encoded middle-T species utilizing enolase as an exogenous substrate. Numbers refer to the levels of pp60$^{c\text{-}src}$ activity relative to the endogenous activity present in neomycin-resistant NIH 3T3 cells.

tions (Fig. 3); however, they encode middle-T species in which only arginine-103 is converted to a tryptophan residue in each case.

When the pBP series were tested in a focus formation assay, pBP1, pBP4, and pBP5 were transformation defective, although able to express a mutated middle-T species, while pBP3 was handicapped in transforming ability, inducing foci at 1/20th the number formed by an equal amount of wild-type plasmid, and pBP2 (which encodes a wild-type molecule) was fully transforming.

The common element of the transformation-defective mutants in the pBP series is the replacement of arginine (103) by either glutamine or tryptophan. Two other arginine residues within the vicinity of residue 103 were mutated in pBP3. This mutant demonstrated a very low transforming capability, even though the basic nature of the residues was conserved (arginine-to-lysine mutations).

Expression of mutant middle-T proteins. To study the middle-T species containing amino-terminal-half mutations, a selection of mutant plasmids were transfected into NIH 3T3 cells together with a plasmid containing the gene for neomycin resistance. Cells resistant to the antibiotic G418 were picked, grown, and analyzed for the presence of a middle-T molecule. The middle-T antigens were labeled, immunoprecipitated with anti-polyomavirus tumor cell serum, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Proteins containing predicted deletions in the cysteine clusters (encoded by pDC1 and pDC2) migrated with very slightly increased mobilities consistent with their expected small lesions. The remaining middle-T species containing point and multiple point mutations in the cysteine-rich regions [pBC1(9), pBC1(nm3), pBC1(nm4), pBC2(22), pBC2(162), and pBC2(163)] comigrated with the wild-type molecule. A similar analysis of the middle-T variants encoded by the SD15 region mutants demonstrated that the proteins migrated with mobilities predicted by their lesions (data not shown).

In vitro kinase activities of amino-terminal-half mutants of middle-T. In vitro kinase assays were done as previously described (5). Middle-T was immunoprecipitated with either rat or hamster anti-polyomavirus tumor cell sera followed by washing and incubation in the the presence of [γ-^{32}P]ATP. Under these conditions, middle-T (hamster serum) or middle-T and the serum immunoglobulin fraction (rat serum) were phosphorylated. The association between middle-T and pp60$^{c\text{-}src}$ was demonstrated by carrying out the in vitro kinase assay after immunoprecipitation with an antibody specific to the proto-oncogene product. To differentiate

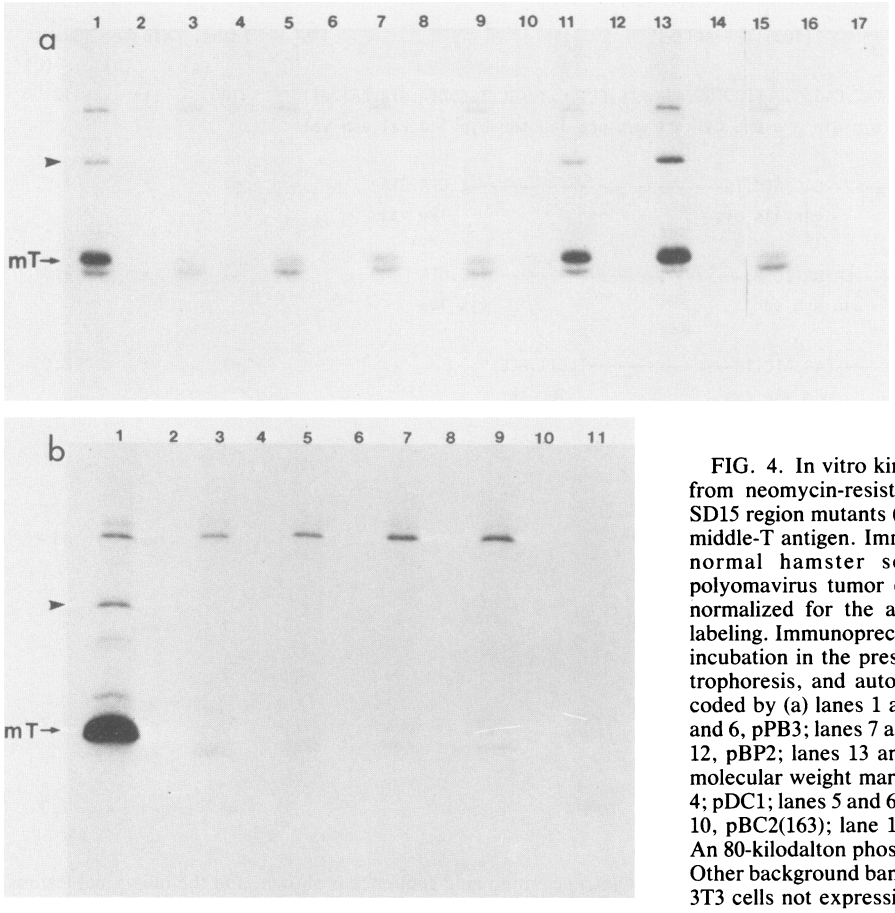

FIG. 4. In vitro kinase (phosphate acceptor) assays with lysates from neomycin-resistant NIH 3T3 cell lines expressing selected SD15 region mutants (a) and selected cysteine region mutants (b) of middle-T antigen. Immunoprecipitates were carried out with either normal hamster serum (even numbers) or anti-hamster polyomavirus tumor cell serum (odd numbers). The lysates were normalized for the amount of middle-T as judged by metabolic labeling. Immunoprecipitates were extensively washed, followed by incubation in the presence of [γ-^{32}P]ATP, polyacrylamide gel electrophoresis, and autoradiography. The middle-T species were encoded by (a) lanes 1 and 2, wild type; lanes 3 and 4, pPB2; lanes 5 and 6, pPB3; lanes 7 and 8, pBP1; lanes 9 and 10, pBP4; lanes 11 and 12, pBP2; lanes 13 and 14, pBP3; lanes 15 and 16, pBP5; lane 17, molecular weight markers; (b) lanes 1 and 2, wild type; lanes 3 and 4; pDC1; lanes 5 and 6, pDC2; lanes 7 and 8, pBC1(nm4); lanes 9 and 10, pBC2(163); lane 11, molecular weight markers. mT, Middle-T. An 80-kilodalton phosphoprotein is also indicated by an arrowhead. Other background bands are also present in neomycin-resistant NIH 3T3 cells not expressing a middle-T species (data not shown).

between middle-T antigen as a phosphate acceptor molecule and as an enhancer of pp60$^{c\text{-}src}$ activity in an in vitro kinase assay, we performed a quantitative assay after immunoprecipitation of the middle-T–pp60$^{c\text{-}src}$ complex by incubation in the presence of [γ-^{32}P]ATP and enolase (which acts as an exogenous substrate).

In vitro kinase assays were done for middle-T species containing lesions in the SD15 region. All the mutants tested displayed some measure of associated kinase activity when immunoprecipitated with anti-hamster polyomavirus tumor serum (Fig. 4a), anti-rat polyomavirus tumor serum (data not shown), or antibodies raised against a peptide sequence of the carboxy terminus of pp60$^{c\text{-}src}$ (data not shown). When lysates from NIH 3T3 cells expressing the SD15 mutant-encoded species were normalized in terms of the amount of middle-T present, the mutant middle-T molecules differed in their phosphate acceptor activities. The middle-T variants encoded by the transformation-defective plasmids (pPB2, pPB3, pBP1, pBP4, and pBP5) displayed a weak activity, while the transformation-handicapped middle-T encoded by pBP3 surprisingly retained wild-type levels of phosphate acceptor activity. Similar results were obtained regardless of the antisera used.

Quantitative kinase assays were done with enolase as an exogenous kinase substrate to determine the ability of each of the SD15 mutant-encoded proteins to activate pp60$^{c\text{-}src}$. The results (Fig. 3) reflect those determined in the other in vitro kinase assays. Lysates containing the pBP3-encoded middle-T activated pp60$^{c\text{-}src}$ some 20-fold above background (being twice that of wild type), while the other transformation-defective species were barely able to activate pp60$^{c\text{-}src}$ above background levels.

When identical assays were carried out with NIH 3T3 cells expressing the cysteine region mutants of middle-T, it was not possible to demonstrate an associated kinase activity or enhancement of pp60$^{c\text{-}src}$ activity for any of the mutants with lesions affecting the cysteine residues. This is shown in Fig. 4b for the cysteine deletion mutants (encoded by pDC1 and pDC2) and the middle-T species encoded by pBC1(nm4) and pBC2(163). These results were confirmed with several different antisera and in a quantitative kinase assay (data not shown).

It is interesting to note that an 80-kilodalton phosphoprotein species is immunoprecipitated with wild-type and transformation-capable species of middle-T but not with nontransforming variants. This protein is phosphorylated only on tyrosine (phosphoamino acid analysis; data not presented) in an in vitro assay and appears to act as a marker for transformation-capable middle-T molecules. A similarly migrating phosphoprotein has also been noted by Matthews and Benjamin (17).

DISCUSSION

This investigation was undertaken in part to extend the mutational analysis of polyomavirus middle-T into the amino-terminal half of the protein and to balance the relative abundance of mutants located in the carboxy-terminal half of the molecule. The NG59 region has been studied previously

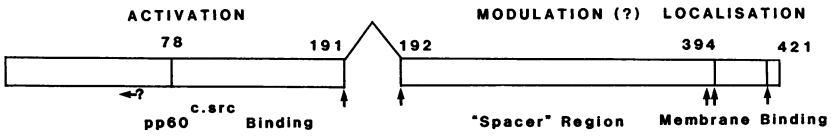

FIG. 5. Schematic diagram of middle-T antigen indicating the three domains discussed in the text. The arrows indicate the suggested boundaries of the domains, while ? indicates the uncertainty of the amino-terminal edge of the domain 1 boundary.

in detail in this laboratory (4), and amino-terminal truncations have also previously been made (14, 25). Mutagenesis of the SD15 region and the cysteine clusters was therefore undertaken to generate mutated species of middle-T which allowed for the further mapping of the amino-terminal half of the protein.

The transforming ability of middle-T is sensitive to mutations in the SD15 region and the cysteine regions. Overall, the mutant middle-T species were predominantly transformation defective; however, they could be separated into two classes as judged by their ability to associate with pp60$^{c\text{-}src}$. The SD15 mutants were mainly nontransforming, although one example (pBP3) retained a marginal transforming activity (approximately 1/20th that of wild type). The interesting feature of the pBP3-encoded middle-T species is that it still retains wild-type levels of associated kinase activity and can activate pp60$^{c\text{-}src}$ to levels normally associated with transformation-capable mutants. Several polyomavirus mutants (13) have been shown to have a similar phenotype. The transformation-defective mutants of this region all demonstrate a weak association with pp60$^{c\text{-}src}$ as judged by in vitro kinase assays. They would appear to be unable to induce transformation owing to their inability sufficiently to activate pp60$^{c\text{-}src}$, as previously described for the pTH-encoded middle-T molecule (16).

The common element of the transformation-defective mutants of the SD15 region is the alteration of arginine-103 to glutamine or tryptophan and its deletion in the pPB series of mutants. A pair of arginine residues is predicted to be present in an analogous position in the hamster papovavirus middle-T. This residue would appear to play a significant role in the interaction between middle-T and pp60$^{c\text{-}src}$. Whether this interaction is dependent on the presence of a basic residue at this position is currently under investigation.

Mutants which encode middle-T species in which the cysteine clusters are either deleted or altered so that at least one of the cysteine residues is changed to a tyrosine residue are all transformation defective. While the cysteine-to-tyrosine alteration is not particularly subtle and the majority of the mutants generated contained multiple mutations, it is worth bearing in mind that multiple mutations have been introduced into several regions of the carboxy-terminal half of middle-T (16, 19) without destroying the transforming ability of the middle-T species. The reason for the defect in the cysteine cluster mutants appears to be an inability to associate actively with pp60$^{c\text{-}src}$, a phenotype identical to that of the original NG59 mutant (3) and certain of the additional mutants made in this laboratory (4). It is possible that the cysteine residues, which are situated in two distinctive clusters, form disulfide bonds; however, other similar cysteine-rich clusters have been noted in nucleic acid-binding proteins which form a so-called "metal-binding finger" (21). Perhaps this region of the middle-T and small-T antigens also forms an important topological feature of significance in their biological roles.

Polyomavirus middle-T antigen can be thought of in simple terms as consisting of three domains, at least in relation to its transforming activity (Fig. 5). One is the putative membrane-binding domain (amino acids 394 to 421), which is important for the correct cellular localization of the protein and possibly also in the interaction with and even the mediation between membrane-bound proteins of significance in the transformation process (discussed in reference 15). Another domain is the first region of middle-T encoded by exon 1 (amino acids 1 to 191) which appears to be the pp60$^{c\text{-}src}$-binding domain and hence involved in the activation of this protein. The third domain is the intervening stretch of amino acids (192 to 393) which may simply be a spacer region, since much of it can be deleted and mutated without total loss of transforming ability. However, certain mutants (for example, pTH [16]) do present a transformation-defective phenotype, implying that this domain plays at least a modulatory role in the transformation process.

Of the four sites within the amino-terminal half of middle-T that have now been studied in detail, all are characterized by a preponderance of transformation-defective point mutants which are either totally or severely handicapped in their ability to associate actively with pp60$^{c\text{-}src}$. The data presented here, together with the results of the amino-terminal deletion mutants (25; D. Cook, personal communication) and the comparative amino acid sequence analyses done in this study imply that at least a large portion (possibly all) of the exon 1-encoded region of middle-T is important for its association with pp60$^{c\text{-}src}$. The striking homology between the amino-terminal halves of the hamster papovavirus and polyomavirus middle-T antigens in terms of both amino acid sequence and predicted secondary structure, in particular the distribution of B turns (data not presented), lends some credence to this hypothesis. It is not known whether the mutations described in this study affect the regions directly involved in the interaction between middle-T and pp60$^{c\text{-}src}$ or simply interfere with the overall conformation of the domain. However, the sensitivity to alteration within the amino-terminal half of middle-T implies that there is a conformational constraint on this portion of the molecule in terms of its interaction with pp60$^{c\text{-}src}$ and, by extrapolation, on its ability to transform.

ACKNOWLEDGMENTS

We thank Rob Harvey for technical assistance, Tom Logan and Doug Lovern for synthesis of oligonucleotides, and Bruce Roberts for critical reading of the manuscript.

W.M. was supported by Public Health Service grant 1 R01 CA43186-01 from the National Institutes of Health.

LITERATURE CITED

1. **Benjamin, T. L.** 1982. The hr-t gene of polyoma virus. Biochim. Biophys. Acta **695**:69–95.
2. **Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A.**

Lipsich, and J. B. Brugge. 1984. Enhancement of cellular src gene product associated tyrosyl kinase activity following polyomavirus infection and transformation. Cell **38**:767–777.
3. **Carmichael, G. G., and T. L. Benjamin.** 1982. Identification of DNA sequence changes leading to the loss of transforming ability in polyoma virus. J. Biol. Chem. **255**:230–235.
4. **Cheng, S. H., W. Markland, A. F. Markham, and A. E. Smith.** 1986. Mutations mapping to the region around the NG59 lesion of polyoma virus middle-T antigen indicate this region is important in $pp60^{c\text{-}src}$ association and cell transformation. EMBO J. **5**:525–534.
5. **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.
6. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein is associated with the product of the c-src cellular gene. Nature (London) **303**:435–439.
7. **Delmas, V., C. Bastien, S. Scherneck, and J. Feunteun.** 1985. A new member of the polyoma virus family; the hamster papovavirus. Complete nucleotide sequence and transformation properties. EMBO J. **4**:1279–1286.
8. **Eckhart, W., M. A. Hutchison, and T. Hunter.** 1979. An activity phosphorylating tyrosine in polyoma T antigen immunoprecipitates. Cell **18**:925–934.
9. **Friedman, T., R. F. Doolittle, and G. Walter.** 1978. Amino acid sequence homology between polyoma and SV40 tumor antigens deduced from nucleotide sequences. Nature (London) **274**: 291–293.
10. **Graham, F. L., and A. J. van der Eb.** 1979. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
11. **Griffin, B. E., and S. M. Dilworth.** 1983. Polyomavirus: an overview of its unique properties. Adv. Cancer Res. **39**:183–268.
12. **Kalderon, D., B. A. Oostra, B. K. Ely, and A. E. Smith.** 1982. Deletion loop mutagenesis, a novel method for the construction of point mutants using deletion mutants. Nucleic Acids Res. **10**:5163–5171.
13. **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. **83**:3624–3628.
14. **Katinka, M., and M. Yaniv.** 1982. Deletions of N-terminal sequences of polyoma virus T-antigens reduce but do not abolish transformation of rat fibroblasts. Mol. Cell. Biol. **2**:1238–1246.
15. **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.
16. **Markland, W., B. A. Oostra, R. Harvey, A. F. Markham, W. H. Colledge, and A. E. Smith.** 1986. Site-directed mutagenesis of polyomavirus middle-T antigen sequences encoding tyrosine 315 and tyrosine 250. J. Virol. **59**:384–391.
17. **Matthews, J. T., and T. L. Benjamin.** 1986. 12-O-Tetradecanoylphorbol-13-acetate stimulates phosphorylation of the 58,000 M_r form of polyomavirus middle-T antigen in vivo: implications for a possible role of protein kinase C in middle-T function. J. Virol. **58**:239–246.
18. **Noda, T., M. Satake, T. Robins, and Y. Ito.** 1986. Isolation and characterization of NIH 3T3 cells expressing polyomavirus small-T antigen. J. Virol. **60**:105–113.
19. **Oostra, B. A., R. Harvey, B. K. Ely, A. F. Markham, and A. E. Smith.** 1983. Transforming activity of polyoma virus middle-T antigen probed by site-directed mutagenesis. Nature (London) **304**:456–459.
20. **Raptis, L., A. L. Boynton, and J. F. Whitfield.** 1986. Protein kinase C promotes the phosphorylation of immunoprecipitated middle-T antigen from polyoma-transformed cells. Biochem. Biophys. Res. Commun. **136**:995–1000.
21. **Rosenberg, U. B., C. Schroder, A. Preise, A. Kienlin, S. Cote, I. Riede, and H. Jackle.** 1986. Structural homology of the product of the Drosophila Kruppel gene with Xenopus transcription factor IIIA. Nature (London) **319**:336–339.
22. **Schaffhausen, B. S., and T. L. Benjamin.** 1979. Phosphorylation of polyoma T antigen. Cell **18**:935–946.
23. **Smith, A. E., and B. K. Ely.** 1983. The biochemical basis of transformation by polyoma virus. Adv. Viral Oncol. **3**:3–29.
24. **Smith, A. E., R. Smith, B. Griffin, and M. Fried.** 1979. Protein kinase activity associated with polyoma middle-T antigen in vitro. Cell **18**:915–924.
25. **Templeton, D., and W. Eckhart.** 1984. N-terminal amino acid sequences of the polyoma middle-sized T antigen are important for protein kinase activity and cell transformation. Mol. Cell. Biol. **4**:817–821.
26. **Tooze, J.** 1981. DNA tumor viruses, 2nd ed., part 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **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.
28. **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.
29. **Yonemoto, W., M. Yarvis-Morar, J. S. Brugge, J. B. Bolen, and M. Israel.** 1985. Tyrosine phosphorylation within the amino-terminal domain of $pp60^{c\text{-}src}$ molecules associated with polyoma virus middle-sized tumor antigen. Proc. Natl. Acad. Sci. USA **82**:4568–4572.