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THE ROLE OF POLYOMAVIRUS MIDDLE T TYROSINES
IN VIRAL INFECTION

presented by

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has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Microbiology and Molecular Genetics

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August 2, 2004

Date

THE ROLE OF POLYOMAVIRUS MIDDLE T TYROSINES IN VIRAL INFECTION

BY

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Molecular Genetics

2004

ABSTRACT

THE ROLE OF POLYOMAVIRUS MIDDLE T TYROSINES IN VIRAL INFECTION

By

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The polyomavirus middle T oncoprotein (MT) plays important roles in the viral lytic cycle. It strongly upregulates both viral early and late gene expression as well as viral DNA replication. MT exerts its functions by binding to and constitutively activating c-Src family tyrosine kinases and their downstream signaling pathways.

In this thesis, I have characterized the role of the "minor" tyrosines Y258, Y288 and Y297 in Src-mediated functions, on their own and in comparison with that of the "major" tyrosines Y250, Y315 and Y322. I examined the effects of tyrosine to phenylalanine MT mutations for their effects on Src binding, Src kinase activity, MT substrate capacity and on MT capability to bind to p85 subunit of phosphatidylinositol-3 kinase. I found that all mutants have the capacity to bind to and activate c-Src. Mutation of both major and minor tyrosines has an impact on Src activity. This decrease in Src activity affects the overall degree of both MT and p85 phosphorylation. Mutation of minor tyrosines reveals a stronger effect when major tyrosines are also removed. In addition, there are tyrosine specific effects in MT substrate capacity and in p85 binding. Y250, Y315, and Y322 have relatively equal substrate capacities, and MT containing a

single active tyrosine Y315, and to a lesser extent, Y250, have the capacity to bind to p85 and induce its phosphorylation.

I have also examined the role of MT C-terminal tyrosines on viral genome accumulation and gene expression in cell culture. Infection of NIH3T3 cells with polyomaviruses expressing different tyrosine mutant MTs demonstrates that a tyrosine sextuple mutant (Y6F) has a severe defect in both viral genome accumulation and gene expression. The major tyrosine triple mutant ($Y3F_M$) has a moderate defect, whereas the minor tyrosine mutant ($Y3F_m$) shows only a minor defect. The capability of different mutant MT to complement the MT^-/ST^- defective virus A185 in genome accumulation and gene expression is also consistent with viral infection data. The MT complementation study also shows that the effect of the major tyrosines is augmenting. Furthermore, the ability of MTs to promote viral DNA replication is reduced but not abolished in the presence of abundant LT. All mutant MTs are also shown to activate polyoma enhancer-origin dependent gene transcription to different degrees.

Finally, I have examined the function of middle T C-terminal tyrosines in viral replication in mice. A tyrosine sextuple mutant (Y6F) shows a 7-10 fold defect in viral genome accumulation in the acute infection of both neonatal and 4-week old mice. This defect becomes more severe in the 4-week persistent infection of neonatal mice. The major tyrosine triple mutant ($Y3F_M$) shows a 2.4 fold defect, whereas the minor tyrosine triple mutant ($Y3F_m$) shows no defect in neonatal mice infection. Furthermore, a mild maturation defect is found in Y6F.

To my wife, Lianjie

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Michele Fluck for her guidance, understanding, support and friendship in the course of my graduate studies. I would like to thank the members of my guidance committee, Dr. Susan Conrad, Dr. Jerry Dodgson, Dr. Sandra Haslam, and Dr. Richard Schwartz for their collective thoughts and invaluable expertise. Finally, although too numerous to mention, I would like to thank all of my friends in the Department of Microbiology and Molecular Genetics, especially in Dr. Fluck's lab, who have offered me enormous friendship and support. Finally, I would like to thank Lianjie, my wife, for her understanding and support through these years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Mouse polyomavirus is a small non-enveloped double-stranded DNA tumor virus. The viral genome consists of 5297 base pairs of closed circular DNA organized as minichromosome and packaged in an icosahedral capsid. The viral genome is divided into early and late regions, separated by a non-coding regulatory region (Figure 1-1, [154]). The viral regulatory region contains two promoters for early and late gene transcription, an enhancer and an origin of replication (Ori). The early gene encodes three viral regulatory proteins - large T (LT), middle T (MT) and small T (ST), which are translated from three differentially spliced transcripts. Large T is a 100-kDa multifunctional nuclear protein that is vital for viral replication, regulation of early transcription [41] and immortalization of primary cells in culture [137, 138]. Middle T is a 56-kDa oncoprotein localized at the plasma membrane. MT is required for cellular transformation [97] and plays an important role in the viral lytic cycle [32] and hence in viral replication and persistence in mice [72]. MT functions are mediated by a series of interactions between MT and cellular signaling molecules involving several tyrosines at the C-terminal half of MT. Small T is a 22-kDa protein located in both the nucleus and the cytoplasm. ST can enhance the function of LT in DNA synthesis [18, 128], complement the function of MT in transformation [14, 126] and tumor induction [12, 13].

The late region encodes three viral capsid proteins – VP1 (45 kDa), VP2 (35 kDa) and VP3 (23 kDa), which are also synthesized from three differentially spliced transcripts.

This thesis will focus on the role of MT C-terminal tyrosines in the activation of MT signaling, viral replication and gene expression during viral lytic infection using cell culture and mouse model. Therefore, this literature review will address the following topics: (1) properties of viral early and late proteins, especially the role of MT in signal transduction and viral lytic cycle; (2) polyoma enhancer and its role in viral replication and gene expression; and (3) polyomavirus lytic cycle, cellular transformation and tumor induction.

PROPERTIES OF POLYOMAVIRUS EARLY PROTEINS

Large T protein

Large T (LT) is a 100-kDa multifunctional nuclear protein. It contains 785 amino acids, with the first 79 amino acids shared by MT and ST. LT plays important roles in initiation of viral DNA replication [49, 187], regulation of early and late gene transcription [68, 100], genome integration [56, 69] and cellular immortalization [58, 66, 74].

LT binds to the polyoma origin as double hexamers. ATP-dependent DNA helicase activity of LT unwinds DNA in the 3' to 5' direction on the DNA strand to which it is bound. LT then forms complexes with cellular proteins, e.g. DNA polymerase α, single strand-specific DNA binding (SSB) proteins, replication protein A (RPA) and topoisomerase I, to initiate DNA replication [67].

LT can also interact with the product of pRb tumor suppressor gene, $p105^{Rb}$ and related proteins, p107 and p130 [55, 65, 89]. The domain responsible for pRb binding and

immortalization is located at the N-terminal of LT between amino acids 141 and 146 [104]. Further study reveals that LT binds to the pRb, p107 and p130 through a common region located between amino acids 141 to 158 [135]. This region contains a consensus pRb binding sequence D/N-L-X-C-X-E. Substitution of any amino acid within this core pRb-binding sequence abolished pRb and p107 binding in vitro and immortalization activity in vivo. The binding of LT to pRb releases transcription factor E2F [188], resulting in the transcriptional activation of E2F controlled cellular genes, including DNA polymerase α, dihydrofolate reductase (DHFR), thymidylate synthetase and thymidine kinase and the subsequent S-phase entry of host cells. The interaction between LT and pRb is also involved in the ability of LT to immortalize primary rodent cells [104]. Mutants containing a single amino acid substitution (Asp-141→ Glu or Glu-146→ Asp) in LT failed to bind pRb and were unable to immortalize primary rat embryo fibroblasts. However in some cases, immortalization function of LT in cell culture is not required for tumor induction in mice [70]. Mutant viruses encoding altered large T proteins that fail to bind pRb, are defective in immortalization of primary rat embryo fibroblasts by a variety of tests, but are capable of transforming both primary and established fibroblasts in culture as well as induce tumors in mice. The DnaJ domain at the N-terminus of large T is required for the regulation of pRb function. Mutations of large T sequences conserved with the DnaJ family affect large T binding to heat shock protein 70 (Hsc 70) [150]. The same mutations abolish large T activation of E2F-containing promoters and pRb binding-dependent large T activation of cell cycle progression. These functions of large T, which cause cells to enter the S-phase are also required for viral DNA replication, presumably as they provide the cellular environment required for DNA synthesis.

p300 and CBP [cAMP-responsive-element-binding factor (CREB)-binding protein] are large nuclear proteins that are involved in transcriptional regulation, chromatin modification and cell cycle control. LT binds to CBP/p300 through its C-terminal region [39, 124]. The QLFP sequence between amino acid 668 and 671 is homologous to the CBP/p300 binding region in adenovirus E1A and SV40 LT. Mutation at residue 671 results in a loss of CBP/p300 binding and of the repressive activity of large T on CREB-mediated transactivation by CBP/p300. These mutants transform rat cells normally but fail to induce a broad spectrum of tumors in mice. Proline 671 substitution mutants grow poorly in normal NIH 3T3 cells but can be partially complemented for growth in mouse cells expressing wild type large T. These findings indicate that CBP/p300 binding by Py large T is essential for virus growth.

Other functions of the C-terminal half of LT comprising approximately residues 264-785 are also studied [78]. This region is sufficient for driving viral DNA replication in vivo in dividing mouse cells using an origin-containing plasmid as a reporter, but is strikingly deficient for replication in serum-starved cells. However, this deficiency can be complemented by coexpression of N-terminal half of LT due to its ability to inducing S-phase entry.

Middle T protein

Middle T's (MT) activities can be observed in both viral growth and transformation. MT is required for high level of viral replication during viral lytic cycle [32, 33]. MT activates polyoma enhancer and increases viral early and late gene expression [40, 51, 117, 190]. The expression of MT is sufficient to transform established

cell lines [97, 138, 167] and to induce multifocal metastatic mammary adenocarcinomas in transgenic mice [87].

Middle T is a 421 amino acid protein with a molecular weight of 56 kDa. MT shares a common N-terminal region (aa. 1-79) with LT and ST, and a further common region with ST (aa. 80-191). There is a stretch of 22 hydrophobic amino acids near its C-terminus that serves as membrane-binding domain. MT lacking this sequence can not bind to cell membrane and does not transform [27]. However, replacement of this hydrophobic region with membrane targeting sequences from vesicular stomatitis virus glycoprotein G [166] fails to maintain transformation while preserves membrane association. In addition, point mutations within this region can also eliminate the transformation ability of MT without affecting membrane binding [112], suggesting that membrane binding is not the only function of this hydrophobic region.

A highly conserved sequence HPDKGG between residues 42 and 47 which is common to all T antigens shares functional homology with J-domain of DnaJ/hsp40 molecular chaperones [99]. J-domain can stimulate ATPase activity of hsc70 and may be involved in the assembly of multi-subunit complexes [156]. Deletion of J-domain in MT does not affect MT transformation [24, 80].

Protein Phosphatase 2A

MT stoichiometrically associates with protein phosphatase 2A (PP2A), a serine/threonine phosphatase. The PP2A core structure is a dimer, consisting of a 36-kDa catalytic C subunit and a 65-kDa regulatory A subunit. A third regulatory B subunit associates with this core enzyme, conferring substrate specificity and subcellular

distribution. Both MT and ST bind to the core dimmer PP2A A+C through their N-terminal region (amino acid 90-120 in MT) [79, 80, 84, 85, 132, 169, 177]. Mutants that do not bind PP2A fail to bind c-Src and to transform. It is not clear at present what other functions are carried out by MT-PP2A association.

c-Src Tyrosine Kinase

MT has no intrinsic enzymatic activity by itself. However, following the binding of MT to PP2A, MT binds to c-Src family tyrosine kinases, including $pp60^{c\text{-}src}$ [47, 48], $pp59^{c\text{-}fyn}$ [38] and $pp62^{c\text{-}yes}$ [102]. Src protein tyrosine kinases are 52-62 kDa proteins composed of 6 distinct functional regions: (1) a short N-terminal Src homology (SH) 4 domain for membrane localization; (2) a unique region for each family member; (3) an SH3 domain, which can bind to proline-rich ligands containing a core consensus sequence of PXXP; (4) an SH2 domain can bind to short continuous amino acid sequences containing phosphotyrosine, and the specificity of individual SH2 domains lies in the 3-5 residues following the phosphotyrosine (+1, +2, +3, etc); (5) a catalytic domain possesses tyrosine-specific protein kinase activity; (6) a short C-terminal negative regulatory tail containing a conserved tyrosine residue.

$pp60^{c\text{-}src}$ was the first protein identified to be associated with MT [47]. Residues 185 to 210 of MT sequence are required for c-Src binding, but have no role in PP2A binding [22, 81]. The activation of $pp60^{c\text{-}src}$ by MT is accompanied both by the dephosphorylation of Tyr 527 [44], a site which negatively regulates $pp60^{c\text{-}src}$ kinase activity and by the autophosphorylation of Tyr 416 [30, 41, 45]. Once activated, MT-associated Src phosphorylates at least three tyrosine residues in the C-terminal region of

MT, Tyr 250 [90], Tyr 315 [96, 130, 145] and Tyr 322 [146]. These phosphorylated tyrosines then provide docking sites for several cellular molecules involved in signal transduction, including ShcA, the p85 subunit of Phosphatidylinositol-3' kinase and PLC-γ1.

Phosphatidylinositol -3 kinase

Phosphatidylinositol-3 kinase (PI3K) was the first signaling intermediate found to bind to MT [46, 184]. PI3K is a heterodimer, consisting of an 85-kDa regulatory subunit (p85) and a 110-kDa catalytic subunit (p110) [28]. The SH2 domain of p85 interacts with phosphorylated tyrosine 315 on MT which contains the preferred PI3K binding motif, YMXM [164, 189]; in turn, p85 phosphorylation stimulates kinase activity of the p110 subunit.

Recently, other studies have reported that p85 can also bind to Y250, although through different mechanisms. The amino acid sequence surrounding tyrosine 250 is NPTYSVM. NPTY is the binding motif for ShcA PTB domain (see below), while YXXM forms a potential binding site for p85. Hong, et. al. reported that Y250 provides a secondary binding site for p85 based on genetic and biochemical analysis [95]. On the other hand, Ong, et al. [129] showed that Y250F shows decreased PI3K activity, which can be restored by insertion of growth-factor-receptor-bound protein 2 (Grb2) binding sites from EGF receptor (YVNQ) or from ShcA (YYND). Since Grb2-associated-binding protein (Gab1) forms a complex with ShcA, Grb2, Ras guanine nucleotide exchange factor son of sevenless 1 (Sos1), and MT [125] as well as other SH2 domain-

containing proteins, e.g. PI3K [94] and PLC-γ1 [86], they suggested that Y250 can indirectly recruit p85 through ShcA-Grb2-Gab1 interaction.

Binding of MT translocates PI3K from cytoplasm to a membrane location where its substrates are present [42]. PI3K phosphorylates the D-3 position of the inositol ring of phosphatidylinositol (4,5)-biphosphate [$PI(4,5)P_2$] to generate inositol 3,4,5-trisphosphate [$PI(3,4,5)P_3$], which can be dephosphorylated by a 5' phosphatase to generate $PI(3,4)P_2$ [149]. These PI3K products bind to the pleckstrin homology (PH) domain of Akt/PKB/RAC, a serine/threonine protein kinase and recruit it to the plasma membrane. $PI(3,4,5)P_3$ and $PI(3,4)P_2$ can also bind to the PH domain of the protein kinase 3-phosphoinositide-dependent protein kinase-1 (PDK-1). Following activation by PI3K, PDK-1 and a less well-defined kinase PDK-2 then phosphorylate Akt at threonine 308 and serine 473, respectively, thus activate Akt [4, 175]. Akt regulate many biological processes, such as proliferation, apoptosis, and growth. Some of the substrates of Akt include the glycogen synthase kinase 3 (GSK3), the ribosomal protein S6 kinase ($pp70^{S6K}$), the transcription factor E2F, and the apoptosis regulating protein Bad.

ShcA [Src homology 2 (SH2) domain-containing transforming protein A]

Phosphorylated tyrosine Y250 of MT was discovered as a binding site for ShcA when a proline 248 to leucine mutation in the NPTY motif of MT was found to cause loss of transformation phenotype, while still able to associate with c-Src and PI3K [25, 59-61]. ShcA is expressed as three isoforms of approximately 46, 52 and 66 kDa. Each ShcA peptide contains an SH2 domain at its C-terminus, a collagen homology region (CH1) and an N-terminal phosphotyrosine binding (PTB) domain. The 66 kDa isoform contains

an additional N-terminal CH-like region (CH2) [139]. ShcA binds to phosphorylated Y250 of MT through its PTB domain and is phosphorylated by MT-c-Src complex at Y239, Y240 and Y317 in the CH1 region. When phosphorylated, these tyrosines can interact with the SH2 domain of Grb2. Grb2 associates through its SH3 domain with Sos1. Relocation of Sos1 to a membrane site activates $p21^{ras}$ [10], which further activates the mitogen-activated-protein kinase (MAPK) signaling cascade.

PLC-γ1

Phosphorylation of Y322 in MT creates a binding site for the SH2 domain of phospholipase C-γ1 (PLC-γ1) [163]. PLC-γ1 is phosphorylated on tyrosines upon association of MT. On activation, PLC-γ1 catalyzes hydrolysis of $PI(4,5)P_2$ to produce the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 provokes a transient increase in intracellular free Ca^{2+}, while DAG serves as a direct activator of protein kinase C (PKC).

14-3-3

14-3-3 denotes a large family of ~30-kDa acidic proteins that exist primarily as homo- and heterodimers within all eukaryotic cells. 14-3-3 proteins interact with phosphoserine in the RSXSXP motif [123]. MT contains this consensus sequence around serine 257 and two 14-3-3 family proteins (27-kDa mixture of several subspecies, and 29-kDa epsilon subspecies) bind to MT through phosphorylated Ser 257 [50, 131], though the kinase responsible for the phosphorylation of Ser-257 is unknown. 14-3-3 has been shown to bind PI3K [21], cdc25 [43] and Raf-1 [108]. 14-3-3 is also involved in the Ras-

dependent activation of Raf-1 [98, 143]. Ser 257 is also involved in MT multimerization [148]. Removal of Ser 257 does not affect transformation of fibroblasts in vitro, since S257A and S257C mutant MTs retained the ability to form foci or colonies in agar, but a virus expressing S257C MT failed to induce salivary gland tumors in mice [50]. The basis for this defect is unknown.

Downstream transcription factors

The downstream targets of MT-induced signaling pathways include transcription factors, including PEA1/AP-1 [147, 181, 182], PEA3/c-Ets [151] [180] and Myc [136].

PEA1 is a mouse homolog of human AP-1. AP-1 family of transcription factors consists of homo- and heterodimers of Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fral, Fra2) or activating transcription factor (ATF2, ATF3/LRF1, B-ATF) bZIP (basic region leucine zipper) group DNA binding proteins [9, 176]. Jun-Jun and Jun-Fos dimers preferentially bind to the phorbol 12-O-tetradecanoate-13-acetate (TPA)-responsive element (TRE; TGACTCA). Three different types of MAPKs, the ERK, the JNK and the p38 MAPK, contribute to the induction of AP-1 activity in response to a variety of extracellular stimuli. c-fos is activated by ERK [35] or ribosomal S6 kinase (RSK) [36] and c-jun is activated by JNK [93].

PEA3 is a mouse homolog of human c-Ets. Ets family transcription factors share an approximately 85-amino acid structurally conserved Ets DNA binding domain. Thirteen subfamilies have been identified [105]. Ets binds to a purine-rich GGAA/T core sequence. Sequences flanking this motif determine the specificity of binding by particular Ets proteins [82].

In the polyoma enhancer, there are PEA1/AP-1 and PEA3/c-Ets binding sites. When activated by MT through signal pathways, PEA1/AP-1 and PEA3/c-Ets can bind to the polyoma enhancer and activate viral DNA replication and gene expression.

Small T protein

Small T (ST) is a 195 amino acid cysteine-rich protein located in both cytoplasm and nucleus. ST binds PP2A [132]. Mullane et. al. showed that although still able to bind to PP2A, a Y250/315F mutant MT did not promote cell cycle progression in serum-starved NIH3T3 cells like ST did. It also failed to activate c-fos whereas ST could. However, this mutant MT was able to activate c-jun, whereas ST was not [119]. Therefore ST-PP2A interaction may have a different role than MT-PP2A interaction.

ST can enhance the function of LT in DNA synthesis [18, 128]. Berger et. al. showed co-expression of ST could partially overcome the replication defect of a mutant polyomavirus genome encoding only LT. Using a dexamethasone-inducible system, Ogris, et. al. further demonstrated that expression of both LT and ST could induce a high percentage of cells to enter S phase, whereas expression of LT or ST alone failed. ST can also complement the function of MT in transformation [14, 126] and tumor induction [12, 13].

Late proteins

The late gene of polyomavirus encodes three capsid proteins, VP1 (45 kDa), VP2 (35 kDa) and VP3 (23 kDa). VP1 is the major capsid protein. The icosahedral capsid contains 72 pentameric capsomers, which each contains five molecules of VP1. VP1 is the only capsid protein exposed on the outside of the virion. The sequence of the minor

capsid proteins, VP2 and VP3 are overlapping. VP2 has 115 amino acids at its N-terminus and contains the entire VP3 sequence at its C-terminus. The highly conserved C-termini of VP2 and VP3 interact with the N-terminus of VP1 [15, 37].

VP1 consists of about six isoelectric species designated A through F, which is caused by post-translational modification [7, 20]. The minor species D, E, and F are phosphorylated, mainly on threonine residues, and to a less extent, on serine residues. Mapping of the phosphorylation sites on VP1 identified in vivo phosphorylation of threonine residues Thr 63 and Thr 156 [106] and in vitro phosphorylation of serine residue Ser 66 [107]. Mutation of Thr 63 does not cause growth defect of the virus, whereas mutation of Thr 156 affect virion assembly. Mutation of Ser 66 does not produce viable viral particles. Casein kinase II can mediate the phosphorylation of Ser 66 on VP1, and is often activated by serum, epidermal growth factor, and insulin-like growth factor [2, 29, 101, 155]. Polyomavirus host range transforming (hr-t) mutants [16] encoding defective MT and ST have been shown to have defect in VP1 threonine phosphorylation at Thr 63 and Thr 156, causing defect in virion assembly [75, 76]. These data suggest a role of MT and/or ST in regulating VP1 phosphorylation.

The N-terminal glycine of the VP2 is myristylated [103, 161]. The removal of the myristyl group by mutating the N-terminal glycine to glutamic acid does not result in a total loss of infectivity, but the efficiency of viral replication is severely diminished.

STRUCTURE AND FUNCTION OF POLYOMA ENHANCER

Polyomavirus enhancer is a 244-bp sequence (nt. 5023-5267) between *Bcl*I and *Pvu*II, located between replication origin and the beginning of late protein coding sequences [53, 54, 168]. It contains DNAse I hypersensitive sites [31] and is devoid of

nucleosomes in 30% of the viral chromatin in infected cells [92, 170], and thus bears characteristics of active chromatin. The polyoma enhancer can be divided into two distinct elements: A domain (*Bcl*I-*Pvu*II, nt. 5023-5130) and B domain (*Pvu*II-*Pvu*II, nt. 5130-5267). The A domain comprises an auxiliary subelement (nt. 5073-5102) and an α-core subelement (nt. 5108-5126). Similarly, B domain comprises two auxiliary subelements, (nt. 5130-5172 and nt. 5202-5218) and a β-core subelement (nt. 5172-5202) [118, 120]. Several binding sites for cellular proteins have been identified, including PEA1/AP-1[113], PEA2 [134, 178] and PEA3/c-Ets [179, 180]. In particular, the α-core of A domain contains juxtaposed binding site for PEA3, PEA1 and PEA2. Many genes activated by signaling through the Ras–Raf–ERK pathway utilize transcriptional control elements containing similar tandem AP-1 and Ets binding sites, e.g. Cyclin D1 [3], the urokinase-type plasminogen activator [170], and interstitial collagenase/matrix metalloproteinase 1 (MMP1) [19]. PEA1/AP-1 and PEA3/c-Ets has been shown to bind to the enhancer [113, 122, 127, 134, 180, 190], leading to the activation of early and late gene transcription [40, 51, 54, 117, 165, 168, 190]. PEA1/AP-1 and PEA3/c-Ets can also bind to polyoma enhancer and directly stimulate DNA replication [11, 122, 172]. PEA1/AP-1 and PEA3/c-Ets are also shown to stimulate DNA replication directly through the bipartite PEA1-PEA3 site in vitro [179-181]. Therefore, polyoma enhancer can regulate viral DNA replication by two mechanisms. (1) The activation of early gene transcription by PEA1/AP-1 and PEA3/c-Ets increases the expression of LT, therefore increases viral DNA replication. (2) PEA1/AP-1 and PEA3/c-Ets can also bind to polyoma enhancer and directly stimulate DNA replication.

The two enhancer domains play different roles in controlling cell-specific transcriptional activation in cell culture [92]. In mouse fibroblasts, element A provides a 3-fold higher enhancement of the alpha 2-collagen promoter than element B. In mouse embryonal carcinoma cells, element B shows the same efficiency as in fibroblasts, whereas that of element A decreases by a factor of 3.5. They also play different role in controlling the age- and organ-specific pattern of viral replication in mice [6].

THE POLYOMAVIRUS LYTIC CYCLE, CELLULAR TRANSFORMATION AND TUMOR INDUCTION

Lytic cycle of polyoma infection

Polyomavirus was first discovered in 1953 by Ludwig Gross as an infectious agent in cell free filtrates from mouse leukemic extracts which caused salivary gland carcinomas when inoculated back into mice [83]. Later this virus was shown to cause a variety of tumors in newborn mice [159], and was subsequently named mouse polyomavirus. It soon became a popular model for studying viral lytic infection, cell transformation and tumorigenesis.

A productive infection of polyomavirus starts with the adsorption of the virion to the surface of permissive cells that support a full lytic infection. The major capsids protein VP1 binds to specific sialyloligosaccharide receptors on the surface of cell membrane [23, 34, 73, 158]. The myristylation of VP2 has also been shown to be important for the infectivity of the virus through an unknown mechanism [103, 144, 161] since VP2 is entirely internal to the virion outer shell. The internalization of virions after the initial adsorption is through monopinocytosis. However, the exact route of polyomavirus entry is still unknown [77], and is unlike SV40 which enters via caveola-

derived vesicles [8, 157], or human JC virus which uses a clathrin-coated pit endocytic pathway [133]. Once uncoated inside the nucleus [110], the viral minichromosome serves as a template for early gene transcription. Pre-mRNA is transcribed from the early region and is spliced into three early transcripts and the early proteins (large T, middle T and small T) are produced. Large T binds to the origin of replication and initiates viral DNA replication. As DNA replication starts, the late promoter is activated and produces the late transcripts. The late mRNA precursor is spliced into three late mRNA, which is translated into capsid proteins, VP1, VP2 and VP3. New virions are assembled and accumulated in the nucleus. When the cells are filled with progeny virions, they lyse and the mature viral particles are released [1].

Virus replication in mice

The course of polyomavirus infection in vivo consists of two distinct phases: acute phase and persistent phase [64]. Following infection of neonatal mice, the level of live virus and viral genome peaks at 7-10 days postinfection [141, 142]. This coincides with the onset of anti-viral immune response [63, 142]. The immune response clears most of the viruses and viral genomes from infected organs within a week, opening the way for the persistent phase. The level of viral genomes gradually decreases, but persists over a long period of time in the kidney, the salivary gland, the bone, the skin and the mammary gland [64, 114, 115, 142, 186]. The overall *de novo* viral replication potential also progressively decreases with the age of the mouse, thus mice infected as neonates produces more viruses than those infected after 3 weeks of age [63, 141, 186]. An organ- and age-specific pattern of viral replication has been observed [57, 63, 185]. In organs such as bone, skin and mammary gland, virus replicates at very high levels in neonates

and at moderate levels in adults, whereas in organs such as kidney, liver and lung, virus replicates at high levels in neonates but at very low levels or not at all in adults. Further study demonstrated that polyoma enhancer plays an important role in determining this age- and organ-specific viral replication pattern [6]. The B domain mediates polyomavirus replication in most organs, in particular in kidney, at neonatal stage but is not required in those organs which can sustain replication in the adult, i.e. mammary gland, skin and bone. The A domain mediates polyomavirus replication at adult stage.

Early studies showed that middle T is essential for virus replication and persistence in mice. For example, a mutant virus PTA-1387T, which encodes a truncated MT lacking the C-terminal membrane insertion region, has a strong detect in viral replication and persistence and fails to induce tumor [72]. The role of MT in viral replication is probably mediated by transcription factors PEA1/AP-1, PEA2 and PEA3/c-ets [113, 134, 165, 179, 182] through polyoma enhancer [33].

The route of infection could also affect the site of persistence. For instance, virus persisted in both lungs and kidneys in an intranasal infection, while only persisting in the kidneys in an intraperitoneal infection [62-64].

Cellular transformation and tumor induction

In tissue culture cells that are permissive to polyomavirus infection, virus replication takes place, followed by the production of progeny virions. It ends with the completion of viral lytic cycle and the lysis of the cells. In cells that are non-permissive to polyomavirus infection, the expression of viral early proteins alters the phenotype of the infected cells, which displays properties of transformation, including loss of contact inhibition and anchorage independence. Some of the short-term changes are also seen in

lytically infected permissive cells. However, the viral genome replicates poorly in nonpermissive cells, due to the inability of LT to interact with the host DNA polymerase-α/primase [121], and, therefore is gradually lost. The transformed phenotype disappears and the cells return to normal growth. This process is called "abortive transformation" [160]. At a low frequency, the viral genome integrates into the host cell genomes at a random site, which results in the continued expression of MT and permanent acquisition of transformed phenotype. The infected cells are transformed by the virus. In cell culture, the expression of MT is sufficient to transform established cell lines [97, 138, 167], but LT is required to transform primary cells [137]. MT Tyr 250 is required for transformation [59, 111]. MT Tyr 315 is not essential for transformation, but mutation of tyrosine 315 to phenylalanine greatly reduces transformation efficiency [26, 116, 130]. Interestingly, expression of MT alone can induce tumors in transgenic mice. In fact, MT is the most potent oncogene tested in transgenic mice.

Polyomavirus infection induces a variety of tumors in mice [52] and MT is required for tumor induction [72]. Three major mouse models have been developed to study tumorigenesis: (1) Neonatal mice infection model; (2) Adult nude mice infection model; and (3) Transgenic mice model.

Infection of neonatal mice leads to a rapid systemic infection. Most of the viruses are cleared from infected tissues following immune maturation, but virus can persist in a lower degree in certain organs throughout the life. Tumor induction occurs later in life. There is a correlation between high genome replication and high tumor incidence, because tumors are developed from organs that support high level of genome replication and persistence in adult mice [186]. The tumor response depends on the genetic

backgrounds of both virus and host. Certain inbred mouse strains are susceptible to tumor induction by polyomavirus. More than 30 cell types can be infected and tumor arises from a subset of these tissues, including the mammary gland, the skin, the bone, the salivary gland and the kidney [52]. Susceptibility in mice is modulated by their MHC type ($H\text{-}2^k$)-related anti-tumor activity and also by an endogenous mouse mammary tumor provirus, Mtv-7. Mtv-7 encodes a superantigen (sag) that delete $V\beta6^+$ T cells required for mounting an effective cytotoxic T lymphocyte (CTL) response to polyomavirus induced tumors [109]. Superantigen-independent susceptibility has also been reported recently [173, 174]. Two wild-derived inbred mice strains, which carry no detectable endogenous Mtv-7 are as fully susceptible to tumor induction by polyomavirus. This superantigen-independent susceptibility is transmitted in a dominant fashion in crosses with MHC-identical immunologically resistant mice [173, 174]. The genetic background of viruses also plays important roles in determining the tumor response. The "high tumor" strain, PTA induces a variety of epithelial and mesenchymal tumors in high incidence and short latency. The "low tumor" strain, RA induces only two types of mesenchymal tumors after a long latent period [52]. The key determinant of PTA in the induction of a high tumor profile is a single amino acid substitution in the major capsid protein VP1 [71].

Infection of adult athymic (nu^-/nu^-) nude mice provides a means to study tumor induction at a specific age and hormonal dependence. In Balb/C mice, tumors appears with a high frequency and a short latency. Tumor induction is restricted to the bone, the salivary gland, the uterus, the skin and the mammary gland [5, 17, 91, 162, 171]. The role of age and ovarian hormone on mammary tumorigenesis has also been demonstrated

[140]. In the ovary-intact mice, the tumor incidence is 100% in mice infected at 3, 6 and 10 weeks of age; it decreases to 55% and 10% in those infected at 20 and 30 weeks of age, respectively. Removal of ovaries 1 week prior to polyomavirus infection reduces the tumor incidence to 61%, 11% and 0% in mice infected at 6, 10 and 20 weeks of age, respectively. Removal of ovary 1 week post infection does not have a significant effect on mammary oncogenesis in 6 week-old mice. Treatment of ovariectomized adult mice with estrogen + progesterone for 1-3 weeks prior to infection was unable to restore tumorigenesis to the level observed in intact mice. These different effects suggest the existence of different status in hormonal responsiveness at these ages, which may correlate with the transition in hormonal responsiveness and mammary gland morphology that occur between these ages.

Transgenic mice expressing polyoma MT has been a widely used model to study mammary oncogenesis [152]. One model system using polyoma MT under the control of MMTV promoter/enhancer demonstrates that expression of polyomavirus middle T antigen results in the widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas and secondary metastatic tumors in the lungs [87]. To further study c-Src in middle T antigen-induced mammary tumorigenesis, a transgenic mouse strain carrying the MMTV/PyV middle T antigen fusion gene and a disrupted c-src gene was generated [88]. In contrast to the rapid tumor progression seen in the original MMTV/PyV middle T antigen strains, mice expressing MT in the absence of functional c-Src rarely develops mammary tumors, suggesting that c- Src tyrosine kinase activity is required for middle T antigen- induced mammary tumorigenesis. The requirement of ShcA and phosphatidylinositol 3' kinase signaling

pathways for polyomavirus middle T-mediated mammary tumorigenesis has also been studied [183]. Transgenic mice that express Y250F or Y315/322F MT developed extensive mammary epithelial hyperplasias. The mammary epithelial hyperplasias expressing Y315/322F MT were highly apoptotic. A proportion of the metastatic tumors arising in mice expressing Y250F MT displayed reversion of the mutant ShcA binding site. These results suggest that polyomavirus MT-mediated tumorigenesis requires activation of both ShcA and PI-3' kinase, which appear to be required for stimulation of cell proliferation and survival signaling pathways, respectively.

SUMMARY

This literature review focused on the MT-induced signaling pathways and the roles of MT in polyomavirus lytic infection and transformation. This study investigates the contribution of C-terminal tyrosines in MT signaling and establishes a model for MT function in viral lytic infection in its natural host. In addition, this study demonstrates a novel role of MT tyrosines in viral replication, providing new insight into the function of MT in virus life cycle.

APPENDIX 1: TABLES AND FIGURES FOR CHAPTER 1

Figure 1-1 Physical map of polyomavirus genome

The inner circle represents the restriction endonuclease sites of MspI on the polyomavirus genome. The early and late coding regions are shown in the outer circles. The coding regions are represented by boxes and the introns are represented by jagged line. The early transcripts are differentially spliced from a common precursor mRNA and translated into the large T, middle T and small T. The late transcripts are processed similarly to produce capsid proteins, VP1, VP2 and VP3. The non-coding viral regulatory region contains the origin of replication, an enhancer, and the early and late promoter. This figure is a modification of the original figure from Soeda et. al. [153].

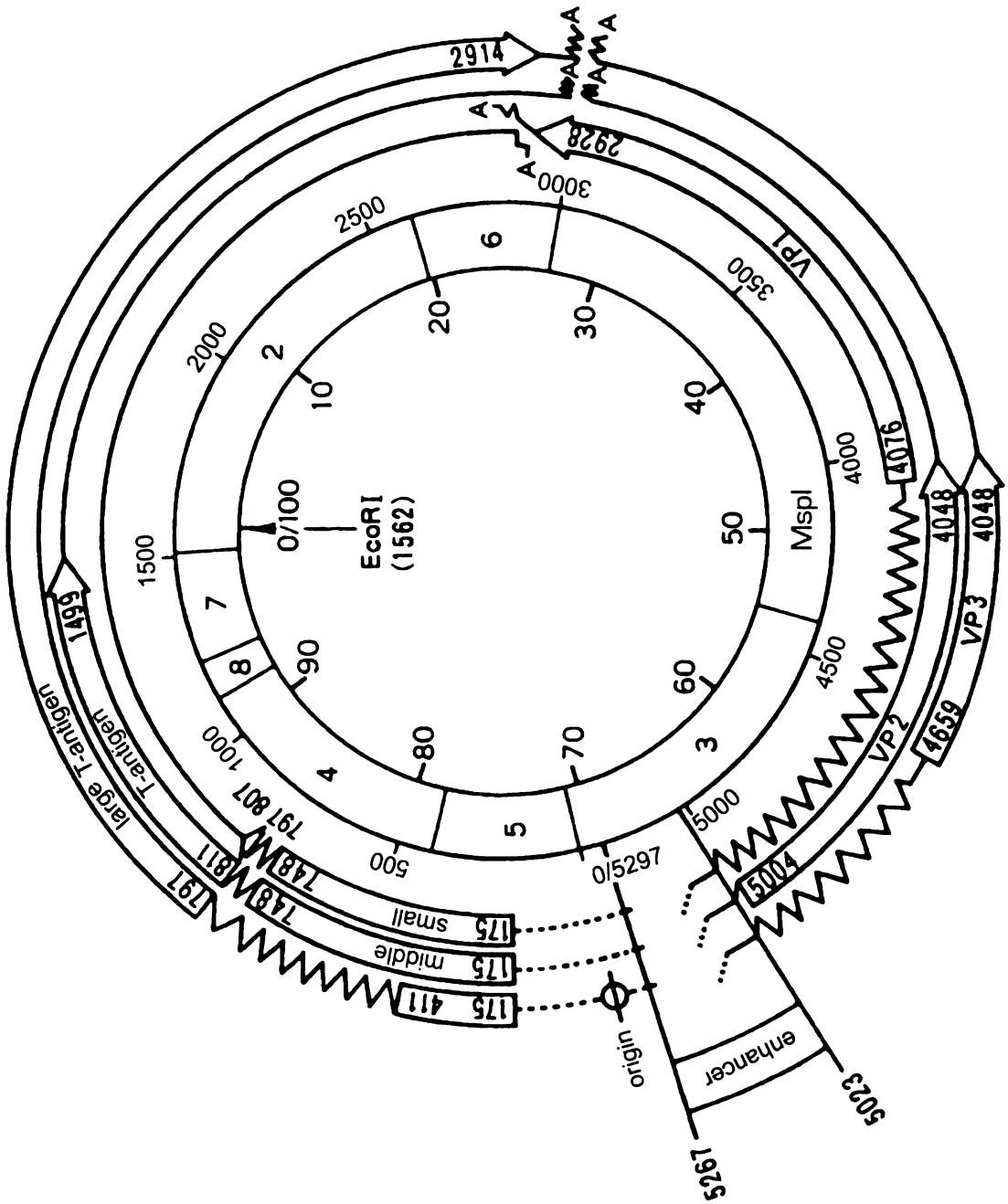

2914
2928
3000
3500
4000
4076
4048
4048
4659
VP3
VP2
VP1
4500
5000
5004
5023
enhancer
origin
5267
0/5297
500
1000
1500
2000
2500
0/100
EcoRI
(1562)
Mspl
1469
large T-antigen
T-antigen
191
807
811
748
748
191
175 small
175 middle
175 411
1
2
3
4
5
6
7
8
10
20
30
40
50
60
70
80
90

Figure 1-2 Lytic cycle of polyomavirus

The lytic infection of cells by polyomaviruses can be divided into early and late stages. The early stage starts with attachment of the virion to cell surface and continues until the beginning of viral DNA replication. This stage is marked by the adsorption and penetration of the virion and its migration to the nucleus, where the viral genome is uncoated and ready for DNA replication and late gene transcription. The late stage begins with the onset of viral DNA replication and ends with release of progeny viruses. This stage includes viral DNA replication, late gene expression, assembly of progeny virions and finally release of viral particles. Multiple steps of a productive polyomavirus infection are depicted. This is a modified version of the figure from Fundamental Virology, 4th ed

Adsorption
Release
Entry
Transport to nucleus
Uncoating
Early transcription
Genome amplification
Assembly
Late transcription
Transport to nucleus
Early translation
T antigens
Late translation
Capsid proteins

Figure 1-3 A schematic representation of middle T

Tyrosines on middle T are presented above the line. The position of DnaJ domain, intron and membrane insertion domain and the binding sites for MT-associated proteins including PP2A, c-Src, ShcA, 14-3-3, PI3K and PLC-γ1 are marked below the line. The signaling pathways activated by these proteins are shown. The common region shared between MT and ST is shown at the bottom.

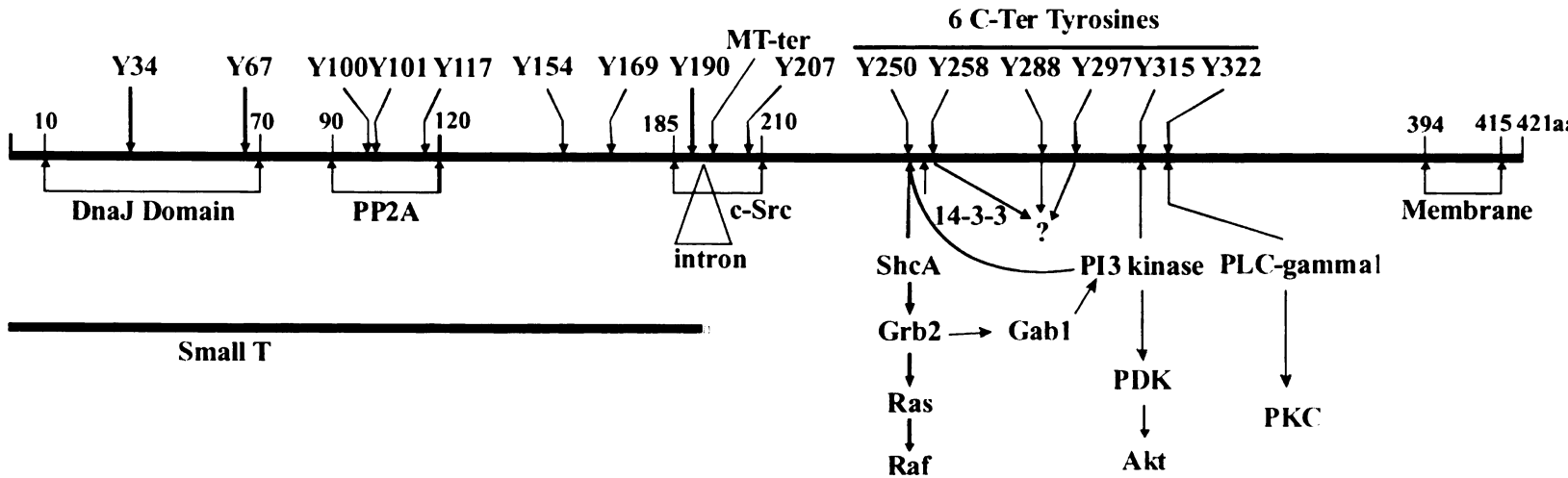

6 C-Ter Tyrosines
MT-ter
Y34
Y67
Y100Y101 Y117
Y154
Y169
Y190
Y207
Y250
Y258
Y288
Y297Y315
Y322
10
70
90
120
185
210
394
415
421aa
DnaJ Domain
PP2A
c-Src
intron
14-3-3
?
Membrane
ShcA
PI3 kinase
PLC-gamma1
Grb2
Gab1
Small T
PDK
Ras
PKC
Raf
Akt

Figure 1-4 Diagram of the signaling pathways mediated by middle T

After binding to cell membrane, MT binds to PP2A and c-Src, leading to the activation of c-Src tyrosine kinase activity. c-Src phosphorylated at least three tyrosines at the C-terminal of MT, including Tyr 250, Tyr 315 and Tyr 322. Binding of ShcA to phosphorylated Tyr 250 recruits Grb2 and SOS, resulting in the activation of Ras. Ras activates MAPK signaling pathways. Binding of the p85 subunit of phosphatidylinositol-3 kinase (PI3K) to phosphorylated Tyr 315 activates PI3K, resulting the activation of PDK1, which further activates Akt. Binding of PLC-γ1 to phosphorylated Tyr 322 activates PKC.

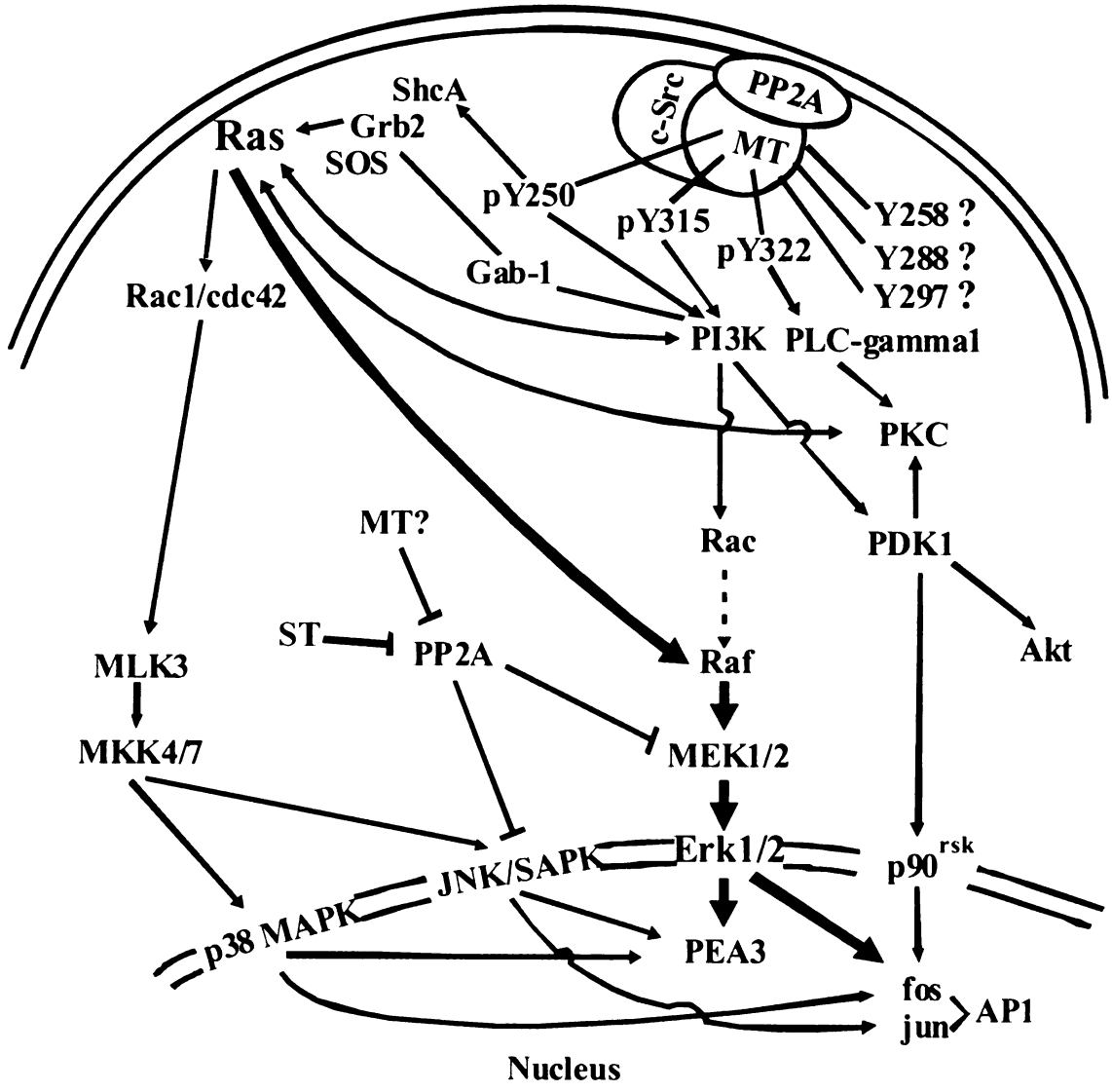
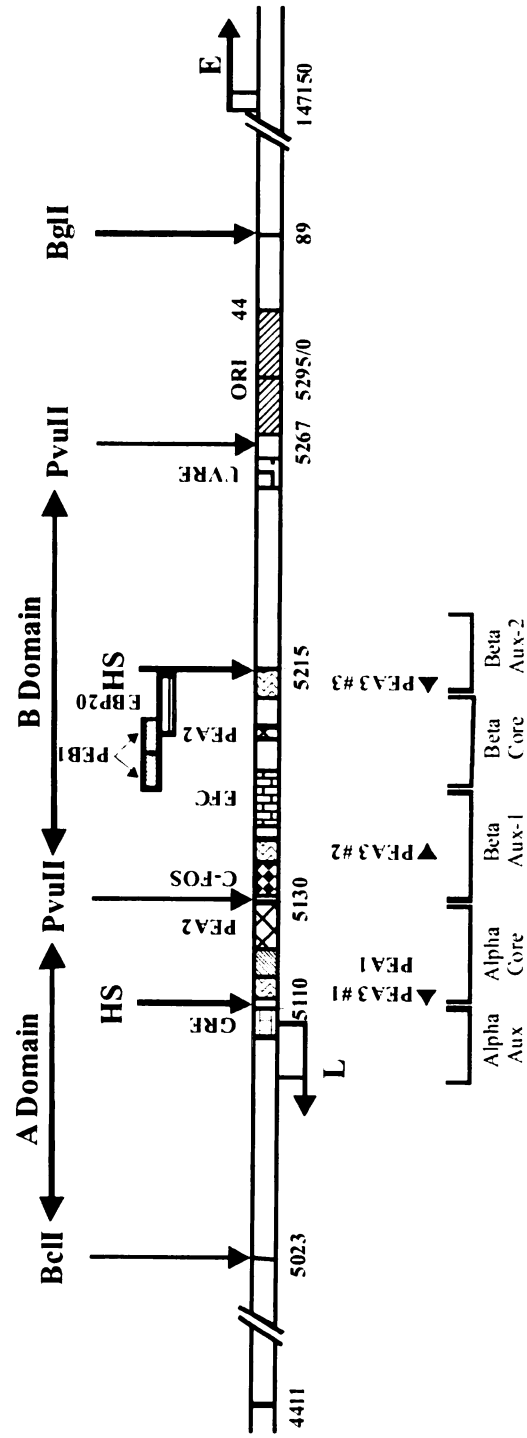

ShcA
Ras
Grb2
SOS
c-Src
PP2A
MT
pY250
pY315
pY322
Y258 ?
Y288 ?
Y297 ?
Gab-1
Rac1/cdc42
PI3K
PLC-gamma1
PKC
MT?
Rac
PDK1
ST
PP2A
Raf
Akt
MLK3
MKK4/7
MEK1/2
Erk1/2
p90 rsk
JNK/SAPK
p38 MAPK
PEA3
fos
jun
AP1
Nucleus

Figure 1-5 Physical map of the enhancer region of polyomavirus A2 genome

The 244 base pair polyomavirus enhancer (BclI-PvuII, nt. 5023-5267) consists of two distinct elements, A domain (BclI-PvuII, nt. 5023-5130) and B domain (PvuII-PvuII, 5130-5467). Each domain can be further divided into a core element and one (A domain) and two (B domain) auxiliary subelements. Cellular transcription factor biding sites are found in the enhancer, including one PEA1 (mouse homolog of human AP1) and three PEA3 (mouse homolog of human c-Ets) sites. The location of polyomavirus origin of replication and the early and late promoters are also indicated. E = Early Promoter; L = Late promoter; HS = DNase I Hypersensitive site.


BclI
A Domain
PvuII
B Domain
PvuII
BglI
E
4411
5023
5110
5130
5215
5267
5295/0
44
89
147150
HS
GRE
PEA2
C-FOS
EFC
PEB1
PEA2
EBP20
SH
LVRE
ORI
L
PEA3 #1
PEA1
PEA3 #2
PEA3 #3
Alpha Aux
Alpha Core
Beta Aux-1
Beta Core
Beta Aux-2

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CHAPTER 2

ROLE OF POLYOMAVIRUS MIDDLE T C-TERMINAL TYROSINES IN MT-MEDIATED SIGNALING

ABSTRACT

The polyomavirus middle T oncoprotein (MT) exerts its functions by binding to and constitutively activating c-Src family tyrosine kinases and their downstream signaling pathways. Previous studies have shown that MT itself is a Src substrate and that C-terminal tyrosines play a crucial role in linking Src activity to cellular signaling intermediates. Tyrosine Y315 was defined as the major Src substrate and shown to bind phosphatidylinositol-3 kinase (PI3K) p85 subunit. Linkage to Ras has been attributed to Y250, which was also shown to play a role in PI3K-dependent signaling. Y322 was found to bind phospholipase C-γ1. In the present studies I have characterized the role of tyrosines Y258, Y288 and Y297 in Src-mediated functions, on their own and in comparison with that of Y250, Y315 and Y322. I examined the effect of tyrosine to phenylalanine (Y→ F) single and multiple MT mutations for their effects on Src binding, Src kinase activity, MT substrate capacity and on MT capability to bind to p85. I found that all mutants, including a sextuple mutant (Y6F) have the capacity to bind to and activate c-Src. Furthermore, in the context of active Y250+Y315+Y322, now defined as "Major" (M) tyrosines, mutation of the "minor" (m) tyrosines 258+288+297 has a very minor effect, even with a triple mutation ($Y3F_m$). I showed that MT carrying a triple major tyrosine mutation ($Y3F_M$) has a residual activity, which is eliminated by mutating the minor tyrosines. Thus, in the absence of active Major tyrosines, the minor tyrosines

have a substantial effect. The data show that mutation of both major and minor tyrosines has an effect on Src activity, as determined by its capacity to phosphorylate enolase. This decrease in Src activity affects the overall degree of both MT and p85 phosphorylation. In addition, there are tyrosine specific effects in MT substrate capacity and in p85 binding. These direct comparisons allowed us to show that Y250, Y315, and Y322 have relatively equal substrate capacities, and that MT containing a single active tyrosine Y315, and to a lesser extent, Y250, (in the context of quintuple mutations of the other M and m tyrosines) have the capacity to bind to p85 and induce its phosphorylation.

INTRODUCTION

The function of polyoma middle (MT) in the virus life cycle and cellular transformation is mediated by the activation of various signaling pathways, in which the interaction between MT and Src family tyrosine kinases plays a central role. The association of MT with these kinases leads to the constitutive activation of their kinase activity and results in the phosphorylation of MT C-terminal tyrosines (Figure 2-1). The major phosphorylated tyrosines Y250, Y315 and Y322 [14, 16, 19, 23, 26] provide binding sites for cellular signaling intermediates, respectively, ShcA [5, 11], the p85 subunit of the phosphatidylinositol 3'kinase (PI3K) [29] and phospholipase C-γl (PLC-γl) [28]. The phosphorylation of these intermediates initiates the activation of three signaling pathways. In addition, there are three other "minor" tyrosines at the C-terminal of MT, Y258, Y288 and Y297, whose functions have not been studied.

To study the functions of MT tyrosines in the polyomavirus life cycle, I mutated the three major tyrosines and found no defect in tissue culture and a minor, if any, replication defect in mice. This unexpected result prompted us to further mutate the

minor tyrosines. The virus expressing a sextuple mutated middle T showed a defect in both tissue culture and in vivo replication. To begin to understand the function of the minor tyrosines, I have examined the characteristics of middle T-Src complexes with a panel of tyrosine mutants. The results show that the phosphorylation of Y250, Y315, Y322, and at least one other tyrosine among Y258, Y288 and Y297 plays a role in the activation of Src tyrosine kinase. I also demonstrate that tyrosine 250 or 315 alone can interact with p85 but both tyrosines are required for efficient binding of p85 to MT.

MATERIALS AND METHODS

Cells, viruses and plasmids

Mouse NIH3T3 fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10% heat inactivated bovine calf serum (GIBCO-BRL) at 37°C and 5% CO_2.

Polyomavirus A2 (WTA2) is the wild-type strain [27]. A185 is a middle T/small T defective mutant, which is derived from WTA2 by site-directed mutagenesis and contains a 71 nucleotide out of frame deletion between nucleotide 646 and 717[18]. The dl45 is a mutant harboring a 66 nucleotide deletion between nucleotide 1075 and 1140, which deletes amino acids 281 to 302 in middle T antigens and also alters large T [1]. MT-ter is a middle T defective mutant with a stop codon inserted after amino acid 196 in MT and therefore only expresses small T. 1387T is a mutant encoding a defective middle T lacking the membrane-anchoring sequence [24]. Middle T C-terminal tyrosine mutants were constructed by site-directed mutagenesis in the A2 background. Tyrosine residues were mutated to phenylalanine by A to T single nucleotide mutations using WTA2 DNA

as template (pUC19-WTA2). Then, polyoma DNA was cut out by *Eco*RI digestion and ligated into a circular genome. The ligated DNA was transfected into NIH3T3 cells using LipofectAMINE 2000 (Invitrogen) to grow live virus. Upon the appearance of cytopathic effect (CPE), the transfection cell lysate was further used to infect NIH3T3 cells. Virus was purified using a standard plaque assay technique and grown in NIH3T3 cells to prepare virus stock. The titer of the virus was calculated using the plaque assay and by comparison of input DNA of a virus infection.

To generate retroviruses that express mutant MTs, the 540 bp *Drd*I-*Eco*RI fragment of mutant polyomavirus DNA was cloned into the PINCO vector [13] containing wild-type middle T cDNA (PINCO-MT/WT) to replace the wild type sequence. The PINCO-MT plasmid was co-transfected into 293T human epithelial cells with a packaging plasmid pSV-ψ2. After 6 hrs of transfection, 10% serum medium was added. Virus stocks were collected every 12 hrs beginning at 60 hrs post transfection. The titers of virus stocks were determined by MT and GFP expression.

All of the tyrosine mutant polyomaviruses and retroviruses were named by the mutations they carried. Their properties with regard to the availability of tyrosines on MT and potential binding sites for cellular proteins ShcA, PI3K and PLC-γ1 are listed in Table 2-1.

Polyoma virus infection

Cells were grown to 50% confluence on 100 mm tissue culture plates followed by 24 hrs of starvation in 0.5% serum medium. After removal of medium, cells were infected with virus at multiplicity of infection of 10 plaque forming units (PFU) per cell

in G_0 state for 2 hrs. The infection lysate was removed prior to the addition of growth serum.

Retrovirus infection

Cells were grown in DMEM supplemented with 10% serum at 37°C and 5% CO_2 until reaching confluence for 24 hrs. Cells were then trypsinized and plated at $8x10^5$ cells/100 mm plate in 10% serum medium at 37°C for 4 hrs. Retrovirus infection was carried out at 37°C for 3 hrs with 8μg/ml of polybrene. The infection lysate was removed prior to the addition of growth medium.

Protein analysis

Infected cells were lysed in Hirt Buffer (10 mM Tris-HCl-10 mM EDTA-0.2% SDS, [pH 7.6]). Proteins were precipitated with 1/10 volume of 5M NaCl at 4°C overnight and collected by centrifugation at 14000 rpm for 30 min at 4°C. Proteins were boiled in 1X protein sample buffer (5% SDS; 0.03% bromophenol blue; 20% glycerol; 5% β-mercaptoethanol; 0.5 M Tris-HCl, [pH 6.8]) for 5 min. Alternatively, cells scraped directly from plates were boiled in 1X protein sample buffer for 5 min. Proteins were electrophoresed in 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Amersham). A polyclonal rat anti-tumor serum harvested as ascites fluid was used as the primary antibody to detect three early proteins: large, middle, and small T. It also reacts with a few cellular proteins which can serve as an internal loading control. Goat anti-rat horseradish peroxidase (HRP; Pierce) was used as the secondary antibody. A rabbit anti-MT antiserum (a gift from B. Schaffhausen) was also used as the primary antibody and goat anti-rabbit HRP (Sigma) as the secondary antibody. A rabbit anti-

capsid antibody, (a gift from R. Garcea) was used to detect the capsid protein VP1, with goat anti-rabbit HRP (Sigma) as the secondary antibody. Signals were visualized by SuperSignal Enhanced Chemiluminescent Detection System (Pierce).

To detect c-Src, a mouse anti-v-Src monoclonal antibody (Oncogene) was used as the primary antibody and goat anti-mouse horseradish peroxidase (American Qualex) as the secondary antibody. To detect active c-Src, a rabbit anti-c-Src pY418 polyclonal antibody (Biosource International) was used as the primary antibody and goat anti-rabbit HRP (Sigma) as the secondary antibody.

Immunoprecipitation and Middle-T-associated kinase assay

The procedure of Schaffhausen and Benjamin was used [25]. Infected NIH 3T3 cells (2×10^6 cells) were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8.0; 100 mM NaF; 10% glycerol; 1 mM $MgCl_2$; 1% NP-40; 1 mM phenylmethylsµlfonyl fluoride, 10 µg of leupectin per ml; 5 µg of aproteinin per ml; 1 mM sodium orthovanadate) and incubated on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 10 min at 4°C. The pellets were removed. The antibodies were added to the supernatant and then incubated at 4°C for 1 hr with rocking. Either the rat polyclonal anti-tumor serum or a mouse monoclonal antibody PAb762 (a gift of S. Dilworth) was used for immunoprecipitation of MT. 50 µl of Protein G-agarose beads (Santa Cruz Biotech) was then added to the supernatant and incubated for 1 hr at 4°C with rocking. The beads were washed twice in 0.5 M LiCl-0.1 M Tris-HCl (pH 6.8) and finally once in 0.2 M Tris-HCl (pH 7.5)-5 mM $MgCl_2$. The immune complexes were suspended in 50 µl of kinase buffer (20 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 20 µCi of [γ-^{32}P] ATP) at 30°C for 30 min. When specified, a Src family kinase selective inhibitor SU6656 (CalBiochem)

was used at 5 μM final concentration. 1 μg of acid-treated rabbit muscle enolase was used per reaction as an indicator of Src kinase activity. To prepare acid-treated enolase, 2 mg of rabbit muscle enolase (Sigma) was mixed with 100 μl of 50 mM Hepes-NaOH buffer, pH 7.0, 1 mM dithiothreitol, 1 mM $MgCl_2$ at 4°C for 10-30 min[8]. Equal volume of 50 mM Acetic acid, pH 3.2 was added at 30°C for 5 min. Finally, 50 μl of glycerol was added. Acid-treated enolase was aliquotted (~10 μg/μl) and stored at -70°C.

Depending on a specific experiment, the kinase products were processed differently following three protocols. (1) The beads were pelleted and washed twice in 0.5 M LiCl-0.1 M Tris-HCl (pH 6.8) and finally once in 1X PBS, 20 mM NaF. The proteins were dissociated from the beads by boiling for 10 min in 1X protein sample buffer (5% SDS; 0.03% bromophenol blue; 20% glycerol; 5% β-mercaptoethanol; 0.5 M Tris-HCl, [pH 6.8]). The supernatant was loaded on a 10% SDS-polyacrylamide gel. (2) When exogenous enolase and Src family inhibitor SU6656 were added to the kinase reaction, 10 μl of 6X protein sample buffer was added to the reaction and boiled for 10 min. Beads were spun down and supernatant was loaded on a gel. (3) If a second immunoprecipitation was required, beads were boiled for 2 min in 400 μl of elution buffer (0.4% SDS, 50 mM Tris-HCl, [pH 7.5], 100 mM NaCl, 2 mM EDTA, [pH 8.0], 2 mM 2-β mercaptoethanol). Iodoacetamide was added to a final concentration of 10 mM. Samples were cooled down, beads were removed by spinning. 1/4 volume of 10% Triton X-100 was added to the supernatant. The second immunoprecipitation was carried out following the same procedure as the first immunoprecipitation. The beads were collected and washed twice in 0.5 M LiCl-0.1 M Tris-HCl (pH 6.8) and finally once in 1X PBS, 20

mM NaF. The proteins were dissociated from the beads by boiling in 1x protein sample buffer. The supernatant was loaded on a gel.

For KOH treatment, the 10% SDS-PAGE gel was rinsed briefly with 1X PBS and then treated with 1N KOH at 55°C for 1 hr. After briefly rinsing again, the gel was soaked in 25% Methanol + 5% Acetic acid for 30-45min. Finally, the gel was rinsed and dried on a vacuum gel drier at 75°C for 1.5 hr and exposed to phosphorescent screens and scanned with the Phosphorimager (Molecular Dynamics). The gel was then exposed to X-ray films (Amersham Pharmacia Biotech) for up to 3 weeks at -70°C with an intensifying screen to achieve darker exposure.

Pulse-Chase Assay

Serum-starved NIH3T3 cells ($8x10^5$ cells /100 mm plate) were infected for 2 hrs with virus at a multiplicity of infection (MOI) of 10 plaque forming units (PFU) per cell and grown in 10% serum medium for 40 hrs at 37°C. Cells were washed with 1X PBS twice, and starved with 1 ml of warm methionine/cysteine free DMEM supplemented with 10% dialyzed calf serum for 1 hr. Then 150 μCi of ^{35}S-methionine (Perkin-Elmer) was added for 2 hrs. After washing cells twice in warm 1X PBS, 10 ml of 10% serum medium with 2 mM methionine was added. Cells were collected at 0 and 4 hrs after chase and proteins immunoprecipitated with the polyclonal rat anti-tumor serum and Protein G-agarose beads. Immunoprecipitates were boiled in 1X protein sample buffer and resolved by 10% SDS-PAGE.

RESULTS

Ability of mutant MT to interact with and activate c-Src

MT C-terminal tyrosine mutants were generated in the PINCO retroviral expression vector [13] as described in Materials and Methods. NIH3T3 cells were infected with different PINCO-MT retroviruses. The expression of MT proteins was examined by Western blotting (Figure 2-2). The levels of MT accumulation at 48 hrs postinfection were high and apparently equal among all the viruses. Similar results were obtained with polyomaviruses carrying the same mutations. The stability of the sextuple mutant Y6F MT was further examined in a pulse-chase experiment. NIH3T3 cells were infected with polyomavirus wild type WTA2, Y6F and a deletion mutant dl45, used to ascertain MT band identity. As shown in Figure 2-3A, similar levels of MT and other viral early proteins were expressed by WTA2, Y6F at the time when the pulse phase started; similar levels were also labeled during the 2 hr pulse period (Figure 2-3B). Furthermore, the level of ^{35}S-labeled wild type and Y6F MT proteins decreased to the same extent over the 4 hr chase. This suggests that multiple Tyr-to-Phe mutations did not change MT stability significantly.

The ability of mutant MT to bind to and activate c-Src was examined. MT was immunoprecipitated from the lysates of NIH3T3 cells infected with retroviruses expressing wild type (WT), the triple mutant in the three major tyrosines ($Y3F_M$), the triple mutant in the minor tyrosines ($Y3F_m$) and the sextuple mutant MT (Y6F). The levels of co-immunoprecipitated c-Src were analyzed by Western blotting (Figure 2-4A). c-Src protein was detected in all samples except that of the control vector, suggesting that

the mutant MTs are still able to bind to c-Src. The levels of c-Src associated with the three mutant MTs appeared proportional to the levels of MT in the precipitates (Figure 2-4C). The MT blot shows two closely spaced bands. The upper band is likely to represent a 58k MT-species with higher Ser/Thr phosphorylation which has been previously shown to be enriched in the MT-c-Src complexes [23]. Association with MT activates c-Src, resulting in auto-phosphorylation of Tyr 418 (Tyr 416 in chicken), which can be demonstrated by using a phosphopeptide-specific antibody [6, 7, 10]. The three mutant MTs were found able to activate c-Src, as detected by the anti-Src pY418 antibody (Figure 2-4B). Thus, the MT, c-Src and active c-Src levels appear proportional to each other, suggesting that mutant MT has no overt defect either in c-Src-binding or in inducing the autophosphorylation of c-Src. However, a small reduction in Src-binding cannot be eliminated by the present experiments.

The activation of c-Src was further examined in an in vitro kinase assay. The immunocomplex associated with MT contains several cellular proteins with similar molecular weight, including MT (56 kDa), c-Src (60 kDa), PP2A regulatory A subunit (60 kDa), which are at stoichiometric levels, ShcA (46, 52 and 66 kDa three isoforms) at low abundance, as well as IgG heavy chain (55 kDa) and enolase (47 kDa) which are added to the kinase reaction. All of these proteins except for PP2A A subunit are phosphorylated by c-Src, thus resulting in a relatively high background. An unknown 50 kDa cellular protein can also be immunoprecipitated by rat anti-tumor serum and phosphorylated. Therefore kinase assay protocols were modified to suit the specific objective of each experiment as described in Materials and Methods. In some early experiments, two rat anti-tumor sera (rat 11 and rat 18) were used for

immunoprecipitation (Figure 2-7A and Figure 2-7B). High background was observed due to the phosphorylation of IgG heavy chain. The high level of IgG also caused distortion of protein bands around the 55 kDa region on the gel. The presence of the phosphorylated 50 kDa cellular protein pulled down by rat antiserum also interfered with the quantitation of enolase phosphorylation. A second immunoprecipitation after the kinase reaction using the same antiserum was performed to clean up the phosphorylated IgG heavy chain and the cellular protein, therefore reducing background around MT. However, double IP also decreases the intensity of the phosphorylation signal due to the loss of phosphoproteins during the second IP. Furthermore, the double IP protocol is not applicable when quantitation of enolase phosphorylation is required, because the second IP requires removing all supernatant after the kinase reaction, which contains phosphorylated enolase. A mouse monoclonal antibody against MT, PAb762 was found to yield better results than the rat antiserum, since the amount of total IgG in the immunoprecipitates can be greatly reduced. This antibody also does not recognize the 50 kDa cellular protein, therefore does not interfere with enolase quantitation. This antibody was used in later experiments (Figure 2-5A and Figure 2-6A, B, C).

The results of a typical experiment are shown in Figure 2-5A. Cells were infected with retroviruses expressing various mutant MTs, resulting in equal levels of MT expression, as determined by Western blotting (Figure 2-5B). MT-c-Src complexes were immunoprecipitated with an anti-T antigen antibody and subjected to an in vitro kinase assay as described in Materials and Methods (Protocol 2) The phosphorylation of enolase, an exogenous substrate for c-Src [8, 17, 20], was measured as an indicator of c-Src kinase activity (Figure 2-5A). As shown, uninfected cells and control vector infected cells

showed no activity. In contrast, wild type and mutant extracts all demonstrated kinase activity, but of differing levels. The level of enolase phosphorylation in the immunoprecipitates containing $Y3F_m$, $Y3F_M$ and Y6F MT decreased to 91%, 50% and 37% of wild type level, respectively. Similar results were found in other experiments; for example, in the experiment shown in Figure 2-6A these levels were 99%, 40% and 23%. This suggests that the ability of MT to activate c-Src is reduced by the tyrosine-to-phenylalanine mutations. The variability from experiment to experiment is presumably due, at least in part, to the relatively high background in this gel region, as discussed above. In experiments in which a second immunoprecipitation was used to further purify the kinase reaction products, higher ratios were obtained between the wild type and $Y3F_M$ and similarly with Y6F mutants (Figure 2-7A, B). When each single major tyrosine alone (i.e. in a quintuple mutant background) was evaluated for its ability to activate Src kinase activity, the pattern showed small differences with Y250> Y315>Y322, as judged by the levels of enolase phosphorylation (Figure 2-6B, C).

To confirm that the MT-associated activity was indeed mediated by a tyrosine kinase, the gel was treated with 1N KOH at 55°C for 1 hr [9]. Alkaline treatment selectively removes serine/threonine-bound phosphate, due to the relative stability of the phosphodiester bond of phosphotyrosine under alkaline conditions [22]. No obvious changes in the phosphorylation pattern was found between gels with and without KOH treatment (Data not shown), indicating that the most of the proteins were phosphorylated at tyrosine residues. However this is not a very stringent method [9]. To further examine the specificity of the kinase reaction, I made use of a selective inhibitor for four of the Src family kinases, Src, Fyn, Yes and Lyn [2, 3]. In the presence of SU6656, the

phosphorylation of most protein species, including enolase, was greatly reduced (Figure 2-5A). This result implies that the enolase and MT phosphorylation, as well as that of other known and unknown visible proteins, is mediated by Src family kinases. No activity was detected in the control vector.

MT as substrate for Src phosphorylation

The capacity of each mutant MT to serve as substrate for Src in vitro phosphorylation was evaluated (Figures 2-5A, 2-6A). To make a quantitative assessment, the mutant specific-Src activity was taken into account by normalizing MT against enolase phosphorylation. Compared to wild type MT, the ratio of MT/enolase phosphorylation for $Y3F_m$, $Y3F_M$ and Y6F MT decreased to 59-89%, 25-35% and 19-18%, respectively, in experiments shown in Figures 2-5A and Figure 2-6A. Similar results were also obtained in transfections with a pMEX-plasmid vector.

The decrease in Y6F middle T phosphorylation suggests that the majority of MT in vitro phosphorylation sites are at its C-terminal half. These ratios also show that Y250, Y315 and Y322 are major phosphorylation sites. Y258, Y288 and Y297 are minor phosphorylation sites on their own, but have a substantially higher in vitro substrate capacity in the absence of phosphorylation at the major sites. The contribution of each major tyrosine 250, 315 and 322 in MT phosphorylation was tested using quintuple mutant MTs (Y5F-250^+, Y5F-315^+ and Y5F-322^+) (Figure 2-6B). The phosphorylation of these three tyrosines was relatively equal, suggesting that these three tyrosines are equal acceptors for in vitro phosphorylation by Src. The quadruple mutants MT (Y4F-$250^+/315^+$, Y4F-$250^+/322^+$ and Y4F-$315^+/322^+$) (Figure 2-6C) showed increased MT phosphorylation close to that of $Y3F_m$ MT.

The study of MT-dependent kinase activity presented so far used a retroviral expression vector to drive MT expression. This activity was compared to that derived during infections with polyomavirus, carrying the same mutations (Figure 2-7A, B). This strategy has the advantage of leading to higher levels of MT expression and hence a higher signal-to-noise ratio in the kinase reaction. In this case, the phosphorylation products were also further purified by a second immunoprecipitation. Polyomaviruses with single or multiple major tyrosine mutations were examined (Figure 2-7A). This experiment was carried out using rat polyclonal antiserum (rat 11) following the protocol (1) described in the legend, in which large T antigen is co-precipitated and phosphorylated [12, 25]. The MT fragment produced by 1387T was also phosphorylated. The intensity of the LT band serves as an indicator of the level of MT in the immunoprecipitate. LT levels are proportional to the levels of active Src. The levels of co-immunoprecipitated active Src were in the same range, except in the case of Y6F and to a lesser extent that of $Y3F_M$, due to slightly lower MT expression. As expected, no Src was pulled down in the case of MT-ter and 1387T, mutants with a termination codon after amino acid 196 and 384, respectively, nor in a WT infection extract immunoprecipitated with a normal control antibody. Loss of each of the major tyrosines, Y250, Y315 and Y322 resulted in a ~2-fold loss in MT phosphorylation in each case, while loss of two of these tyrosines caused a 4-6-fold drop; the bigger drop for the Y250/322F double mutant is likely to reflect at least in part the lower level of active Src in this extract. Loss of two minor tyrosines (Y288 + Y297) in the dl45 deletion mutant resulted in a 2.5-fold loss in MT phosphorylation. As shown in Figure 2-7A, despite the co-expression of the other early polyoma proteins, large T and small T antigens, the

pattern obtained is qualitatively very similar, except for the presence of phosphorylated LT. The low expression of Y6F MT was corrected in another experiment (Figure 2-7B). In this case, a 25-fold difference in MT phosphorylation was observed between wild type and Y6F MT, even though higher MT expression was obtained in the Y6F infection (due to slightly off dosage).

Based upon Western blotting results, the band above MT in these electrophoregrams of kinase products (e.g. Figure 5A) corresponds to the c-Src position. I assume that its low level of in vitro phosphorylation is reflective of its in vivo phosphorylated status, given that only MT-complexed c-Src and hence only active, phosphorylated c-Src is pulled down by anti-MT antibodies.

Requirement of MT tyrosines for PI3K binding

The role of the major tyrosines in the recruitment p85/PI3K to the MT-c-Src complex was examined indirectly, by way of p85 phosphorylation in the kinase assay. For this purpose, tyrosines 250, 315 and 322 were tested individually in quintuple mutants and in pairs in quadruple mutants to determine if single tyrosines are which is sufficient for p85 binding. The results of in vitro kinase assays are shown in Figures 2-5A and Figure 2-6 (panel A, B and C), and Figure 2-8A, in which the p85 position is indicated by an arrow. The size of the phosphorylated ~85 kDa band was determined with molecular weight markers, and matches the migration of the p85 subunit of PI3K determined using anti-p85 antibody in a Western blot run in parallel. As previously demonstrated, Y315F mutation resulted in the loss of most of the p85 signal, a smaller reduction was observed with Y250F mutation and Y322F mutation had no effect. The triple mutation of the minor tyrosines also had no effect. The concomitant mutation of

Y315 and Y250 totally abolished the p85 signal, as shown in the immunoprecipitates from cells expressing Y5F-322+, Y3FM and Y6F middle T (Figure 2-6A, B). In a corollary analysis, quintuply mutated MT that contained only Y315 showed the capacity to bind p85, as seen with Y5F-315+; this Y315 capacity is also seen with Y4F-315+/322+ (Figure 2-6C). Binding was also detected, albeit to a lower level, in the case of Y5F containing only Y250, as well as with Y4F-250+/322+ (Figure 6B and 6C long term exposure). With both Y250 and Y315 intact, mutant MTs were able to bind p85 as efficiently as wild type MT, as seen with Y4F-250+/315+, Y3Fm, dl45, and Y322F. The presence of Tyr 322 or that of the minor tyrosines did not facilitate p85 binding. The difference in the levels of MT-bound phosphorylated p85 may be the result of changes in both p85 binding to MT or in Src kinase activity. However, with similar or even lower kinase activity judged by enolase phosphorylation, the levels of phosphorylated p85 in samples expressing Y5F-315+ and Y4F-315+/322+ MT were always higher than those expressing Y5F-250+ and Y4F-250+/322+ MT, suggesting that these differences are not likely due to lower Src kinase activity. The comparison of the levels of bound phosphorylated p85 is summarized in Table 2-2.

DISCUSSION

In this study, the effect of tyrosine mutations on the capacity of MT to bind to and activate Src tyrosine kinase and the capacity of these tyrosines to serve as phosphate acceptor for Src in vitro phosphorylation were examined. Three tyrosines, Y258, Y288 and Y298, termed minor, that had not been tested previously in details were tested, and the well studied tyrosines Y250, Y315 and Y322, termed major, were reexamined in this

context. Two new conclusions can be drawn from this work. Tyrosine mutations were shown to impact Src enzymatic activity, while having no detectable effect on Src binding. Furthermore, the minor tyrosines were found to play a substantial role in modulating Src activity, which could be revealed only in the absence of the major tyrosines.

MT six carboxyl-terminal tyrosines were studied in parallel by tyrosine to phenylalanine mutations, all generated in the A2 genetic background. The minor tyrosines were studied as a block, either in the presence of functional major tyrosines by comparing WT to $Y3F_m$, or in their absence, in comparisons of $Y3F_M$ and Y6F. In contrast, the major tyrosines were studied one at a time, either as single mutations in the context of functional other tyrosines (Y1Fs), or as single functional tyrosines in the context of quintuple mutations (Y5Fs). This study represents the most comprehensive direct comparison to date. All mutants were found to be stable, and normal in their capacity to bind to c-Src and to induce its autophosphorylation [6]. This point was established in comparisons of MT immunoprecipitates of wild type, $Y3F_m$-, $Y3F_M$-, and Y6F-MT expressing extracts, analyzed by western blotting with antibodies against MT and total c-Src. In each case, the Src levels were proportional to MT levels. Furthermore, the levels of activated Src probed with an antibody directed against Src- phosphopeptide spanning Tyr 418 were proportional to the level of total Src. These results are not surprising, since previous studies of double mutants Y250/315F, Y315/322F have not uncovered defects in Src-binding and since binding to MT activates Src, resulting in autophosphorylation. However, when Src enzymatic activity was tested on a heterologous substrate, enolase, in an in vitro kinase assay, the mutations of the three major tyrosines led to a reproducible 2-2.5-fold decrease in enolase phosphorylation. In contrast, triple

mutation of the minor tyrosines did not lead to a decrease in Src activity, in the context of three intact major tyrosines; however, a 1.5-2-fold decrease was observed when the minor tyrosines were mutated in the context of absence of the major tyrosines. Thus Src complexed to the sextuple Y6F MT shows an overall 3-4-fold decrease in activity compared to wild type. The restoration of single major tyrosines into the Y6F background led to an increase in activity, further increased by restoring two major tyrosines. The contrast between the normal capacity for autophosphorylation of Src complexed with multiply mutated MT and the decreased capacity for heterologous phosphorylation is intriguing. It may reflect differences in the assay: the autophosphorylation test measures a steady state following an undetermined, but likely long in vivo interaction time, while enolase phosphorylation was detected in a 30-minute reaction. Since the c-Src binding site has been mapped to MT to amino acid 185 to 210 [4], which is not altered by the tyrosine mutations, the exact mechanism for this regulatory effect of MT on c-Src activity is unknown at present. I note that an effect of tyrosine mutation on Src activity on enolase was suggested in early studies [19]. These experiments reported large differences based on studies of Y250 and Y315 mutations. It is possible that these different results were due to using an MT transformed cell line in their study instead of using polyomavirus and retrovirus infection.

The enolase phosphorylation test revealed a function of the minor tyrosines in this process that was not detectable in the presence of normal major tyrosines. A similar situation has been documented in the case of phosphorylated Y315, whose major, detectable role is to bind p85 subunit of PI3K, which provides a consensus binding motif for p85 via one of its SH2 domain [15]. However, when the p85 binding motif is altered,

phosphorylated Y315 appears to bind an unknown partner, which participates in a signaling that contributes toward transformation. Similarly, Y322 may help in p85 binding, while it participates in PLCγ-1 binding in the absence of p85 binding [30]. As will be discussed below, the minor tyrosines are a weak Src substrate. Thus it is conceivable that their contribution toward Src-activity takes place in the absence of phosphorylation. However, it is equally possible that these minor tyrosines are only phosphorylated in the absence of major tyrosine phosphorylation, and that MT phosphorylation is at the root of enhancing Src activity, in addition to creating phosphotyrosine- and SH2-binding sites. The residual function of $Y3F_M$ MT may be mediated by the binding of one or more of the minor tyrosines to signaling intermediates, or by Src trans-kinase activity.

The capacity of MT tyrosine mutants to serve as Src substrates was also determined. To normalize for reduced Src activity, the ratio of MT/enolase phosphorylation was calculated for each mutant tested. This strategy also corrects for unavoidable occasional differences in MT levels. The loss of phosphorylation caused by each major Y→F mutation in the wild type context was examined, as well as the contribution of single major tyrosine in a quintuple mutant context. The results confirm previous findings, that the major tyrosines are primary substrate sites while the minor tyrosines are weak substrate sites. Nevertheless, the phosphorylation of one or more among the minor tyrosines is clearly detectable. Evidence for Y297 phosphorylation has previously been presented [16]. Our results with dl45 deletion mutant, which encompasses Y288 and Y297, are in agreement. In studies of single major tyrosine mutations I found that the three major tyrosines are equally good substrates for the in

vitro kinase. This was a consistent finding. It was demonstrated both with a MT retroviral vector and in polyoma viral infections when analyzing single mutants, as well as in the context of quintuple mutants with a single functional tyrosine (retroviral vector only). Additionally the behavior of double mutants also supports this observation. The results suggest a simple augmenting pattern. A number of previous reports have suggested Y315 as the primary phosphorylation site in vitro as well as in vivo [23] and Y250 as another primary site in vitro [16], whereas Y322 as a secondary site in vitro [26]. Several factors might be accounted for the observed differences, such as precise conditions used for cell growth and infection, antibodies used for immunoprecipitation, etc.

The in vitro kinase electrophoregrams easily demonstrate the presence of the p85 subunit of the PI3K; the band intensity reflects the combination of levels of bound protein and phosphorylation efficiency. The results confirm previous findings that pY315 provides a major binding site [29] and pY250 a minor site. Recent studies showed that tyrosine 250 can recruit p85 directly [15] or indirectly through Grb2-Gab1 interaction [21]. Experiments with the quintuple mutants demonstrate that Y315 alone and to a lesser extent Y250 are able to bind p85, and that a quadruple mutant containing both Y250 and Y315 binds as efficiently as the wild type.

The other MT-Src partners were not reliably detected in these electrophoregrams. A faint band corresponding to Src size was seen in wild type and Y3Fm extracts. As mentioned in the results section it is expected that most of the Src protein found in association with MT is activated and thus phosphorylated in vivo, as suggested by the results of blots with the antibody directed against the Src activation site. A band

corresponding to the position of PLC-γ1 was observed in very active extracts of cells infected with wild type and dl45 polyomavirus; it was absent in Y6F. Its intensity was considerably lower than that displayed by p85. This may reflect competition between PI3K and PLC-γ1 for binding in the Y315/Y322 region.

SUMMARY

In summary, the present experiments have revealed that the mutations of MT tyrosines affect the activity of the MT-associated Src kinase, and that the minor tyrosine Y258, Y288 and Y297 play a role in this MT function. The effect of tyrosine mutations on Src-enzymatic activity affects MT phosphorylation and thus is likely to impact all aspects of MT signaling, via decreases in binding of the MT-associated signaling intermediates. A strong biological support for the role of the minor tyrosines was obtained in the infections of mice, in which the replication and survival of polyomavirus strains carrying the Y6F mutation were shown to be poorer than that of a strain the $Y3F_M$ mutation (See Chapter 4).

APPENDIX 2: FIGURES AND TABLES FOR CHAPTER 2

Figure 2-1 A schematic representation of middle T

Tyrosines on middle T are presented above the line. The position of DnaJ domain, intron and membrane insertion domain and the binding sites for MT-associated proteins including PP2A, c-Src, ShcA, 14-3-3, PI3K and PLC-γ1 are marked below the line. The signaling pathways activated by these proteins are shown. The common region shared between MT and ST is shown at the bottom.

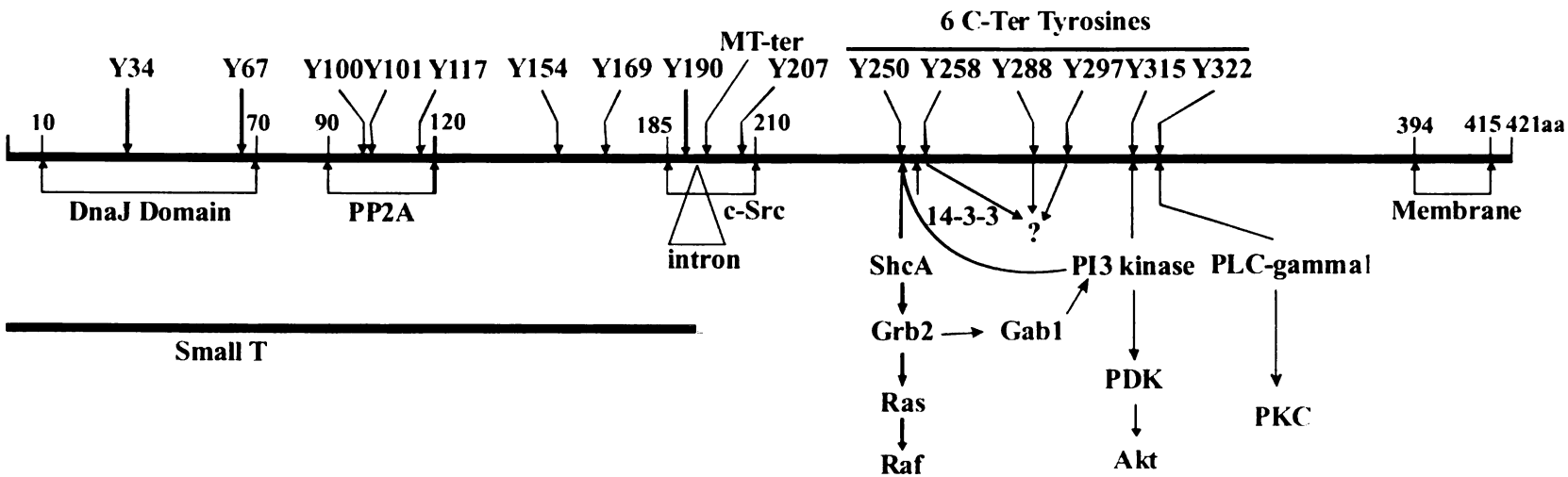

6 C-Ter Tyrosines
MT-ter
Y34
Y67
Y100Y101 Y117
Y154
Y169
Y190
Y207
Y250
Y258
Y288
Y297Y315
Y322
10
70
90
120
185
210
394
415
421aa
DnaJ Domain
PP2A
c-Src
intron
14-3-3
?
Membrane
ShcA
PI3 kinase
PLC-gamma1
Grb2
Gab1
Small T
PDK
Ras
PKC
Raf
Akt

Table 2-1 Tyrosine mutant middle T

This table lists the tyrosine mutant MTs used in this study. Six C-terminal tyrosines were mutated to phenylalanine in various combinations to generate single, double, triple, quadruple, quintuple and sextuple mutant MT. Tyrosines 250, 315 and 322 are referred to as the major tyrosines and tyrosines 258, 288 and 297 as the minor tyrosines. The capability of each mutant MT to bind ShcA, PI3K and PLC-γ1 are indicated.

Mutations	Viruses	Available C-terminal tyrosines	Binding sites for cellular proteins		
			ShcA	PI3K	PLC-γ1
Wild Type	WT	250, 258, 288, 297, 315, 322	+	+	+
Single Mutants	Y250F	258, 288, 297, 315, 322	-	+	+
	Y315F	250, 258, 288, 297, 322	+	-	+
	Y322F	250, 258, 288, 297, 315	+	+	-
Double Mutants	Y250/315F	258, 288, 297, 322	-	-	+
	Y250/322F	258, 288, 297, 315	-	+	-
	Y315/322F	250, 258, 288, 297	+	-	-
Triple Mutants	$Y3F_M$	258, 288, 297	-	-	-
	$Y3F_m$	250, 315, 322	+	+	+
Quadruple Mutants	$Y4F\text{-}250^+/315^+$	250, 315	+	+	-
	$Y4F\text{-}250^+/322^+$	250, 322	+	-	+
	$Y4F\text{-}315^+/325^+$	315, 322	-	+	+
Quintuple Mutants	$Y5F\text{-}250^+$	250	+	+	-
	$Y5F\text{-}315^+$	315	-	+	-
	$Y5F\text{-}322^+$	322	-	-	+
Sextuple Mutant	Y6F	-	-	-	-

Figure 2-2 Analysis of tyrosine mutant middle T expression

Cells were infected with retroviruses expression different tyrosine mutant MTs as described in Materials and Methods. Proteins were extracted at 72 hours postinfection. The Western blot was probed with a polyclonal rat anti-tumor serum.

MT
Vector
WT
Y3F$_M$
Y3F$_m$
Y6F
Y5F-250$^+$
Y5F-315$^+$
Y5F-322$^+$
Y4F-250$^+$/315$^+$
Y4F-250$^+$/322$^+$
Y4F-315$^+$/322$^+$

Figure 2-3 Pulse-chase analysis of the stability of Y6F middle T

Cells were infected with polyomavirus WTA2, Y6F and dl45. The dl45 virus has a deletion in MT from amino acid 281 to 302, which was included as a reference to identify MT protein. Cells were infected as described in Materials and Methods. (A) A Western blot of early proteins at 40 hrs postinfection. (B) In the meantime, cells on duplicated plates were pulse-labeled for 2 hrs and chased for 4 hrs. Proteins were collected immediately before and after the chase phase. The three viral early proteins were immunoprecipitated with the polyclonal rat anti-tumor serum, and subjected to reducing SDS-PAGE.

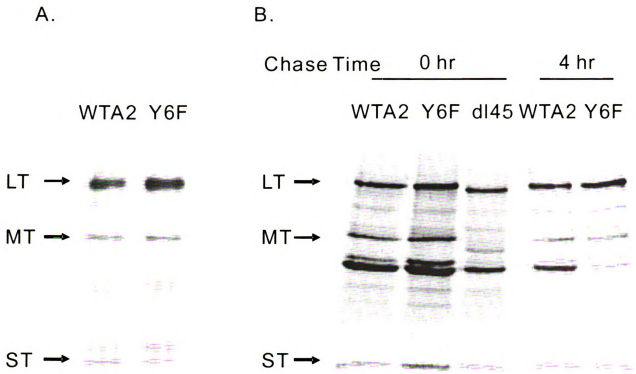

A.
WTA2 Y6F
LT
MT
ST
B.
Chase Time 0 hr
4 hr
WTA2 Y6F dl45 WTA2 Y6F
LT
MT
ST

Figure 2-4 Analysis of c-Src activation in cells expressing mutant MTs

Cells were infected with retroviruses expressing different tyrosine mutant MT. Proteins were extracted at 40 hrs postinfection and immunoprecipitated with a mouse monoclonal anti-T antibody (PAb762). The Western blot was probed sequentially with (A) a mouse anti-v-Src antibody; (B) a rabbit anti-c-Src pY418 antibody; and (C) a polyclonal rabbit anti-T serum.

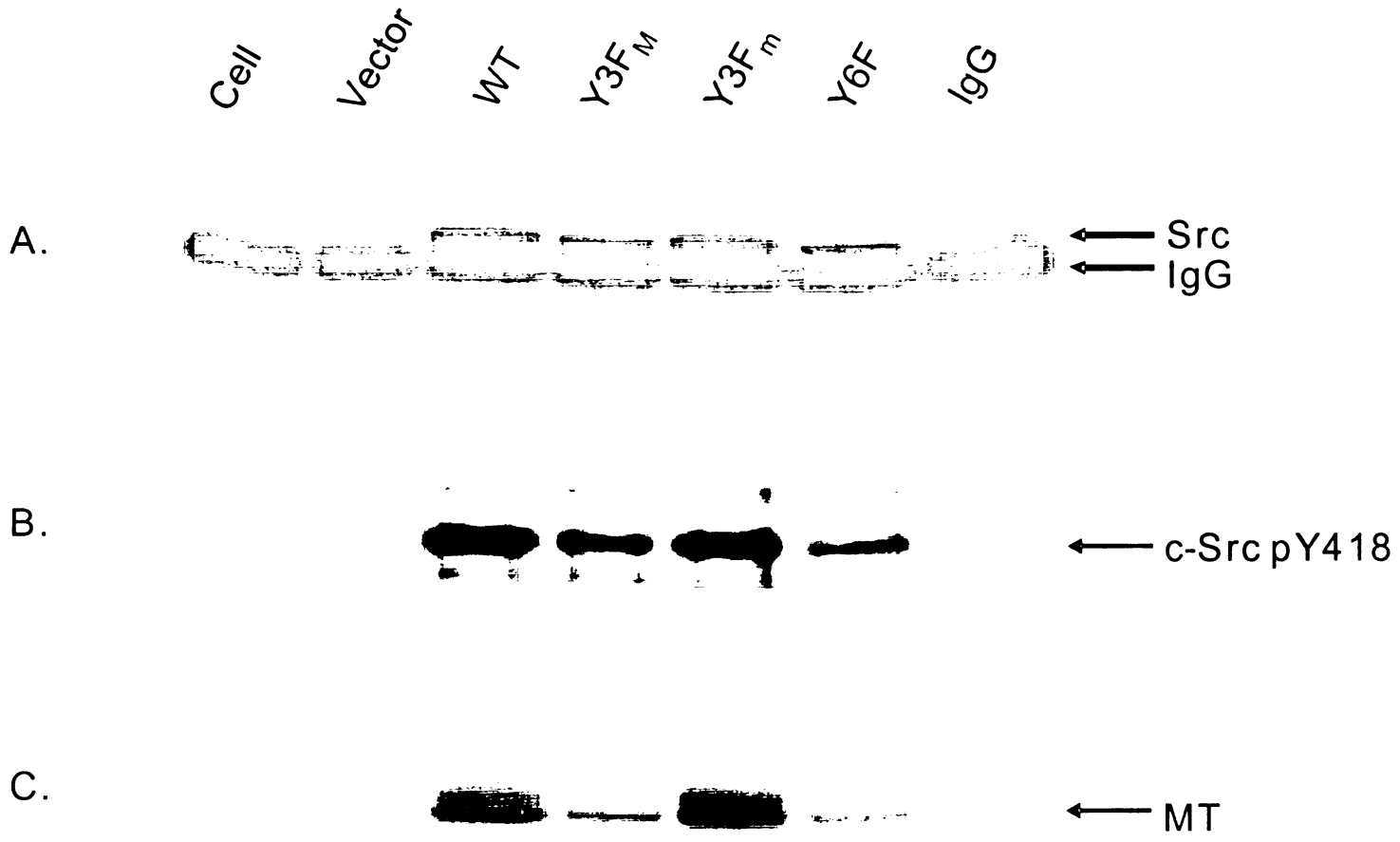
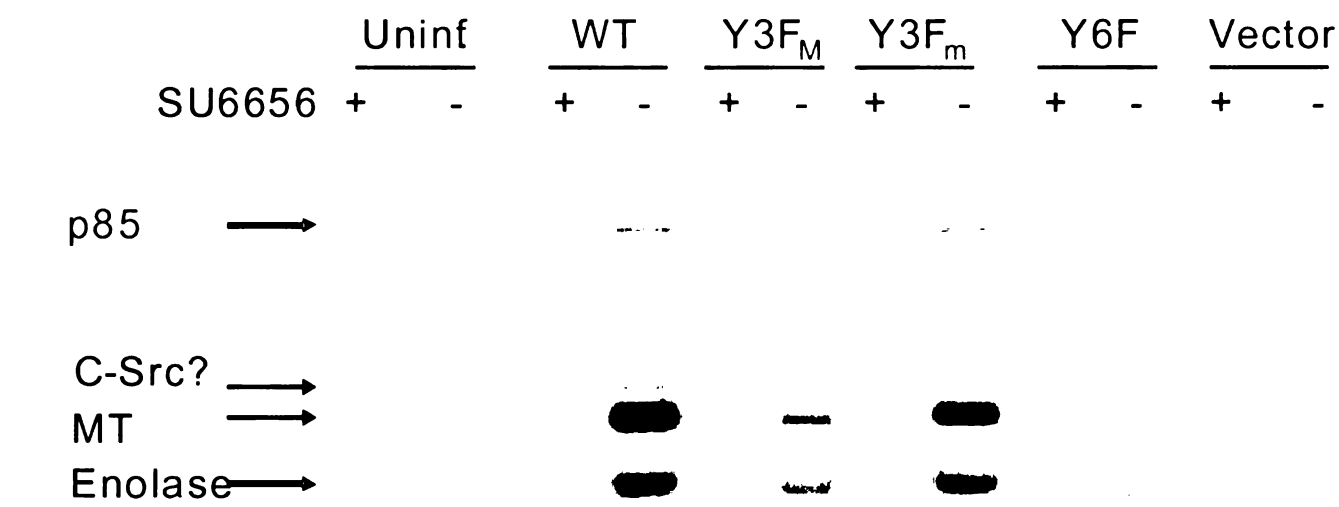

Cell
Vector
WT
Y3F M
Y3F m
Y6F
IgG
A.
Src
IgG
B.
c-Src pY418
C.
MT

Figure 2-5 MT-associated in vitro kinase assay and the effect of Src inhibitor SU6656

Cells were infected with retroviruses expressing different mutant MTs as described in Materials and Methods. Proteins were extracted at 40 hrs postinfection. (A) In vitro kinase assay. A total of 2 x 10^6 cells were immunoprecipitated with the monoclonal antibody (PAb762), and a kinase assay was performed as described in Materials and Methods (Protocol 2). An uninfected cell control and a vector control were also immunoprecipitated at the same time. A Src family selective inhibitor SU6656 was added to the duplicated samples. An exogenous Src substrate, enolase was added as an indicator of Src kinase activity. The bands representing phosphorylated middle T, enolase and the p85 subunit of PI3K are indicated. The intensity of phosphorylated enolase and MT were counted. The counts of enolase as well as the ratio of counts between MT and enolase were calculated and compared to that of wild type MT. (B) A Western blot of proteins from cell lysates (1/20th of total cells). This blot was probed with the rat polyclonal serum. MT is identified with an arrow.

A.



Mutant/WT Ratio

Enolase(%)	100	50	91	37
MT/Enolase(%)	100	25	59	19

B.

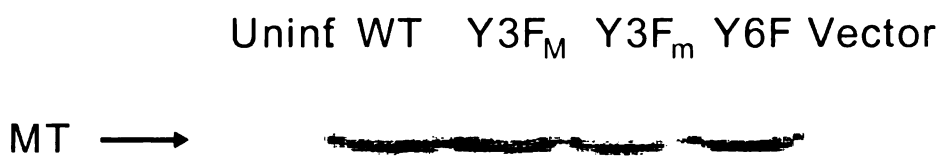
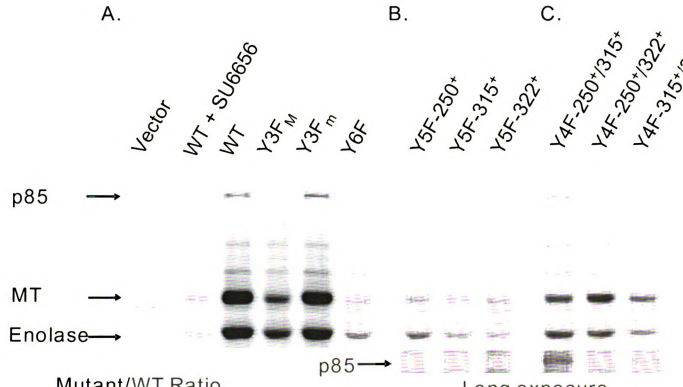


Figure 2-6 Analysis of Src activity and MT phosphorylation by MT-associated in vitro kinase assay

Cells were infected and proteins extracted as described in Materials and Methods. In vitro kinase assay was performed as described in the legend to Figure 2-5. The bands representing phosphorylated middle T, enolase and the p85 subunit of PI3K are indicated. The relative ratio of enolase and MT phosphorylation of mutant/WT middle T are calculated. (A) Vector, WT+ Src inhibitor SU6656, WT, $Y3F_M$, $Y3F_m$ and Y6F; (B) Tyrosine quintuple mutants Y5F-250^+, Y5F-315^+ and Y5F-322^+; (C) Tyrosine quadruple mutants Y4F-$250^+/315^+$, Y4F-$250^+/322^+$; Y4F-$315^+/322^+$. A two-week exposure of p85 in panel (B) and (C) is shown underneath

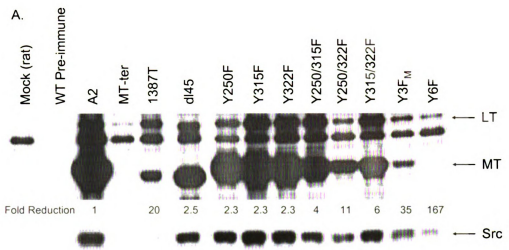


Mutant/WT Ratio

Enolase (%)	100	40	99	23	25	14	14	46	34	25
MT/Enolase(%)	100	35	89	18	30	33	49	42	77	56

Figure 2-7 MT phosphorylation in cells infected with polyomaviruses by in vitro kinase assay

Cells were infected as described in Materials and Methods. Proteins were extracted at 40 hrs postinfection. In vitro kinase assay. A total of 2 x 10^6 cells were immunoprecipitated with the polyclonal antiserum, and an in vitro kinase assay was performed, followed by a second immunoprecipitation as described in Materials and Methods (Protocol 3). The bands representing phosphorylated large T, middle T, p85 and cellular protein are indicated. The reduction of MT phosphorylation of mutants compared to wild type A2 is presented. (A) Cells were infected with different mutant polyomaviruses. Rat polyclonal antiserum (rat11) was used for immunoprecipitation. An uninfected cell control precipitated with immune serum (Mock) and A2 infected cell precipitated with pre-immune serum were included. A Western blot of extracts of the same infected cells was probed with anti-c-Src p418 antibody is shown at the bottom. Given its size, similar to dl45 MT, the band in 1387T infected sample is assumed to be MT 1387T fragment. This experiment was performed by Dr. Li Chen. (B) Cells were infected with WTA2, Y6F and dl45. Rat polyclonal antiserum (rat18) was used for immunoprecipitation. An uninfected cell control was precipitated with immune serum. The bands representing phosphorylated large T, middle T and p85 are indicated. A Western blot of proteins from cell lysates (1/20th of total cells) was probed with the rat polyclonal serum. MT is identified with an arrow.


A.
Mock (rat)
WT Pre-immune
A2
MT-ter
1387T
dl45
Y250F
Y315F
Y322F
Y250/315F
Y250/322F
Y315/322F
Y3F$_M$
Y6F
LT
MT
Src
Fold Reduction
1
20
2.5
2.3
2.3
2.3
4
11
6
35
167

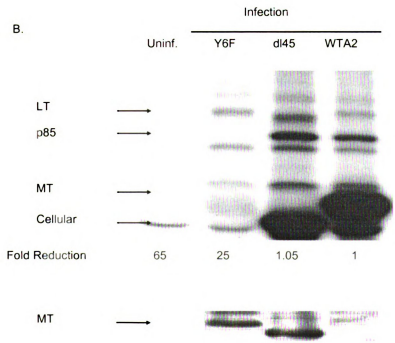

B.
Infection
Uninf.
Y6F
dl45
WTA2
LT
p85
MT
Cellular
Fold Reduction
65
25
1.05
1
MT

Figure 2-8 Requirement of MT tyrosines for p85 binding

Cells were infected with different polyomavirus mutants as described in Materials and Methods. Proteins were extracted at 40 hrs postinfection. (A) In vitro kinase assay was performed as described in the legend to Figure 2-7. Only the bands representing phosphorylated p85 are presented. (B) A Western blot of proteins from cell lysates (1/20th of total cells). This blot was probed with the rat polyclonal serum. MT is identified with an arrow.

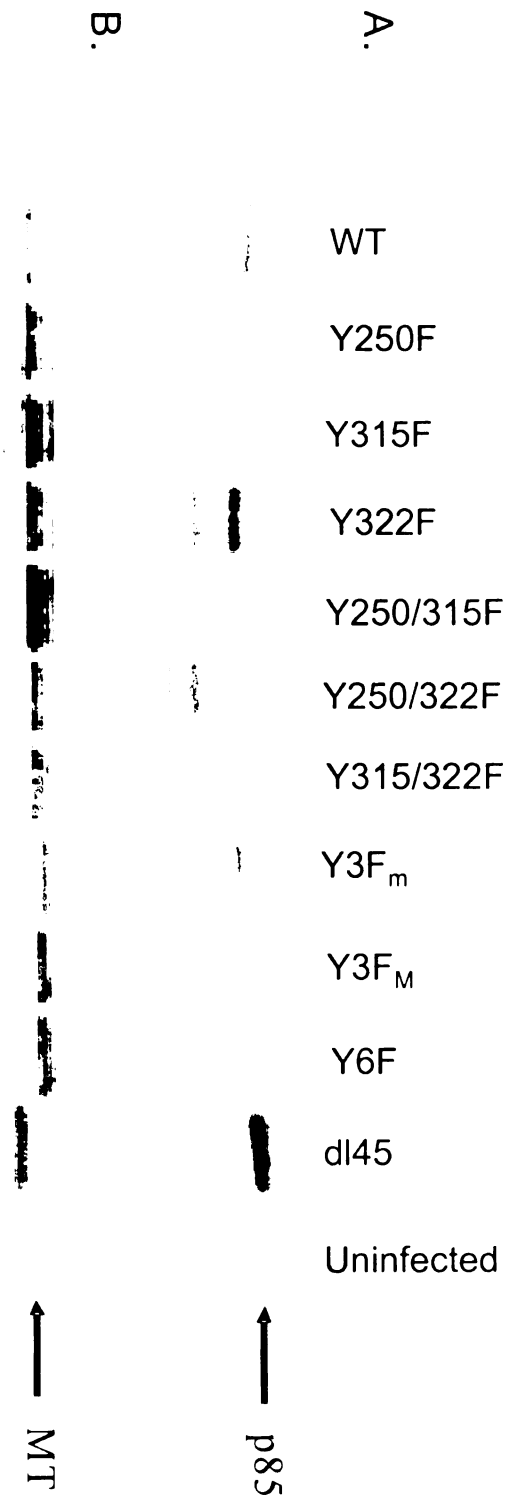

A.
B.
WT
Y250F
Y315F
Y322F
Y250/315F
Y250/322F
Y315/322F
Y3F$_m$
Y3F$_M$
Y6F
dl45
Uninfected
MT
p85

Table 2-2 Summary of the levels of phosphorylated p85 in the in vitro kinase assays

The levels of phosphorylated p85 were compared among different mutant MTs. Given the variation in the levels of MT expression, the relative levels of p85 were adjusted accordingly. Results from retrovirus infection (Figure 2-6A, B and C) and polyomavirus infection (Figure 2-8A) are summarized.

Retrovirus	p85 Level	Polyomavirus
WT	++++	WT
$Y3F_m$	++++	$Y3F_m$
$Y4F\text{-}250^+/315^+$	++++	Y322F
$Y4F\text{-}315^+/322^+$	++	Y250F
$Y5F\text{-}315^+$	++	Y250/322F
$Y4F\text{-}250^+/322^+$	-/+	Y315F
$Y5F\text{-}250^+$	-/+	Y315/322F
$Y5F\text{-}322^+$	-	Y250/315F
$Y3F_M$	-	$Y3F_M$
Y6F	-	Y6F

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CHAPTER 3

THE ROLE OF MT C-TERMINAL TYROSINES IN VIRAL DNA REPLICATION AND GENE EXPRESSION IN CELL CULTURE

ABSTRACT

I have been studying the effect of middle T on viral DNA replication in NIH3T3 cells. Here I describe the role of MT C-terminal tyrosines on viral genome accumulation and gene expression in cell culture. Infection of NIH3T3 cells with polyomaviruses expressing different tyrosine mutant MTs demonstrates that a tyrosine sextuple mutant (Y6F) has a severe defect in both viral genome accumulation and gene expression. The major tyrosine triple mutant ($Y3F_M$) has a moderate defect, whereas the minor tyrosine mutant ($Y3F_m$) shows only a minor defect. The capability of different mutant MT to complement the MT^-/ST^- defective virus A185 in genome accumulation and gene expression is also consistent with viral infection data. The MT complementation study also shows that the effect of the major tyrosines is augmenting. Furthermore, the ability of MTs to promote viral DNA replication is reduced but not abolished in the presence of abundant LT. Finally, I show that all mutant MTs can activate polyoma enhancer-origin dependent gene transcription to different degrees.

INTRODUCTION

Polyomavirus MT plays a major role in viral lytic cycle: it strongly upregulates the expression of both early and late genes [7, 10, 18, 31], as well as DNA replication [5, 6]. Most of these functions are thought to be mediated by transcription factors, i.e.,

PEA1/AP-1 and PEA3/c-Ets [16, 25, 31] through the activation of the viral enhancer. By increasing LT expression, which is ultimately responsible for viral DNA replication, MT indirectly promotes viral DNA replication. PEA1/AP-1 and PEA3/c-Ets have been shown to bind to the polyoma enhancer and directly stimulate DNA replication [1, 19, 30], therefore suggesting another mechanism for MT regulation of viral DNA replication. MT C-terminal tyrosines 250, 315 and 322 are involved in MT mediated signaling and are able to activate different signaling pathways leading to the activation of AP1 and PEA3/c-ets. This function is likely to represent the requirement for MT in the lytic cycle.

Here, I investigate the involvement of these tyrosines in the viral lytic cycle and demonstrate the role of these major tyrosines in viral DNA replication and gene expression in cell culture. I also expand the study to three minor C-terminal tyrosines 258, 288 and 297 for their potential roles in MT function. The results from this study suggest that these six tyrosines are involved in MT regulation of viral DNA replication and gene expression. Tyrosines 250, 315 and 322 play a major role whereas tyrosines 258, 288 and 297 play only a minor role.

MATERIALS AND METHODS

Cells, viruses and infections

Polyomavirus WTA2, $Y3F_M$, $Y3F_m$, Y6F, MT-ter and A185 have been described previously. NIH3T3 cells were grown to 50% confluence on 35 mm tissue culture plates followed by 24 hrs of starvation in 0.5% serum medium. After removal of medium, cells were infected in the G_0 state for 2 hrs. The infection lysate was removed prior to the addition of 0.5% serum medium.

Retroviruses expressing various mutant MTs have been described previously. NIH3T3 or NIH3T3-LT (stably expresses LT) cells were grown in DMEM with 10% serum at 37°C and 5% CO_2 until reaching confluence for 24 hrs. Cells were then trypsinized and plated at $2x10^5$ cells/35 mm plate in 10% serum medium at 37°C for 4 hrs. Retrovirus infection was carried out at 37°C for 3 hrs with 8μg/ml of polybrene. The infection lysate was removed prior to the addition of 0.5% serum medium.

Preparation and analysis of DNA

Infected cells were lysed at 6 hrs and 48 hrs postinfection in Hirt Buffer (10 mM Tris-HCl-10 mM EDTA, 0.2% SDS, [pH 7.6]). Proteins were precipitated with 1/10 volume of 5M NaCl at 4oC overnight. Proteins were isolated for Western blot analysis by centrifugation at 14000 rpm for 30 min at 4oC. DNA was extracted with phenol-chloroform. DNA was digested with the restriction endonuclease EcoRI (Gibco-BRL), which linearizes the polyomavirus genome. Digested DNA was electrophoresed on 1% agarose gels, stained with ethidium bromide, and blotted onto nylon membranes (Amersham Pharmacia Biotech). Hybridization was carried out at 65oC in 1X Denhardt's solution, 2X SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7]) by standard procedures with a 32P-radiolabeled probe containing the whole polyomavirus genome. Hybridization probes were labeled with [α-32P] dCTP using random primer DNA labeling kit (Invitrogen). The hybridized blots were washed under stringent conditions. The blots were exposed to phosphorescent screens and scanned with a Phosphorimager (Molecular Dynamics).The blots were then exposed to X-ray films (Amersham Pharmacia Biotech) for up to 3 weeks at -70oC with an intensifying screen to achieve darker exposures.

Protein analysis

Proteins from Hirt extraction (see DNA analysis) or cells scraped directly from plates were boiled in protein sample buffer (5% SDS; 0.03% bromophenol blue; 20% glycerol; 5% β-mercaptoethanol; 0.5 M Tris-HCl, [pH 6.8]) for 5 min. Proteins were electrophoresed in 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Amersham). A polyclonal rat anti-tumor serum, harvested as ascites fluid, was used as the primary antibody to detect three early proteins: large, middle, and small T. It also reacts with a few cellular proteins which can serve as an internal loading control. Goat anti-rat horseradish peroxidase (HRP; Pierce) was used as the secondary antibody. A rabbit anti-MT antiserum (a gift from B. Schaffhausen) was also used as the primary antibody and goat anti-rabbit HRP (Sigma) as the secondary antibody. A rabbit anti-capsid antibody, (a gift from R. Garcea) was used to detect the capsid protein VP1, with goat anti-rabbit HRP (Sigma) as the secondary antibody. Signals were visualized by SuperSignal Enhanced Chemiluminescent Detection System (Pierce).

Luciferase reporter gene assay

EOL cells are a stable NIH3T3 cell line that contains an integrated luciferase gene under the control of the polyomavirus enhancer-origin. EOL cells were grown in DMEM with 10% serum at 37°C and 5% CO_2 until reaching confluence for 24 hrs. Cells were then trypsinized and plated at $2x10^5$ cells/35 mm in 10% serum medium at 37°C for 4 hrs. Retrovirus infection was carried out at 37°C for 3 hrs with 8μg/ml of polybrene. The infection lysate was removed prior to the addition of 2% serum medium for 18 hrs. Growth medium was changed to 0.5% for 48 hrs. Cells were washed with cold 1x PBS and lysed in 150 μl of 1x reporter lysis buffer (Promega). Samples were mixed by

vortexing for 15 sec and centrifuged at 14000 rpm for 2 min at 4°C. 20 µl of the extract was assayed for luciferase activity with 100 µl of Luciferase reagent (Promega) using a TD-20e luminometer (Turner).

RESULTS

Effect of MT tyrosine mutations on viral genome accumulation during polyomavirus infection

I first studied the effect of different tyrosine mutant polyomaviruses in viral lytic infection. NIH3T3 cells were infected with wild type A2 (WTA2) or $Y3F_M$, $Y3F_m$, Y6F, MT-ter and A185. The mutant properties with respect to the expression of early viral proteins are shown in Table 3-1. Input and output viral DNA was collected at 6 and 48 hrs post infection, respectively. Viral DNA was analyzed by Southern blotting.

During 48hrs of infection, the WTA2 genome amplified 900 fold, $Y3F_M$ 100 fold, $Y3F_m$ 400 fold, Y6F 130 fold; MT-ter 33 fold and A185 8 fold (Figure 3-1). Thus, relative to WTA2, Y6F showed a 7-fold, $Y3F_m$ a 2.5-fold and $Y3F_M$ a 9-fold defect in genome accumulation. The slightly stronger defect of $Y3F_M$ compared to Y6F is likely due to variations in DNA dilution and loading, since the accumulation of $Y3F_M$ genomes in other experiments was usually similar or slightly higher that that of Y6F. A185 showed a more than 100 fold defect in genome accumulation, consistent with published data [5]. MT-ter had a 30 fold defect compared to WTA2, but had a 4-fold increase compared to A185. Thus small T makes a small and MT large contribution to the amplification of the viral genome This result confirmed the role of MT [5] [12]and ST [2, 20, 29] in viral

DNA replication. Interestingly, the defect of Y6F was less severe than that of MT-ter, which suggested that residuals other than the six tyrosines on MT were involved in regulating viral genome accumulation. The 2.5-fold defect of $Y3F_m$ indicated a new role of the three minor tyrosines (tyrosines 258, 288 and 297) in MT function. However, these tyrosines play only a minor role in viral genome accumulation since $Y3F_m$ had a weaker defect than $Y3F_M$.

Effect of MT tyrosine mutations on viral gene expression during polyomavirus infection

The effect of MT tyrosine mutations on viral early and late gene expression was examined. NIH3T3 cells were infected with WTA2 or $Y3F_M$, $Y3F_m$, Y6F, MT-ter and A185. Viral early and late protein expression was analyzed by Western blotting (Figure 3-2). Compared to WTA2, the expression of LT in A185 was much lower, indicating the requirement of MT and ST for efficient early gene expression. In all other mutants, the expression of LT was only slightly reduced. However, the pattern showed WT = $Y3F_m$ > Y4F > Y5F $\geq Y3F_M$ > Y6F. Levels of MT and ST in WTA2, $Y3F_M$, $Y3F_m$ and Y6F were also only slightly different. As expected, MT-ter only expressed ST. Larger differences were observed in the level of late protein VP1. $Y3F_M$ showed decreased VP1 expression to a level similar to Y6F, while the level of VP1 in $Y3F_m$ was similar to that in A2. These results suggested that Y6F and $Y3F_M$ MT are defective in upregulating viral late gene expression, while $Y3F_m$ MT was not. Therefore, as they did in genome accumulation, the major tyrosines also played a more important role in regulating viral gene expression than the minor tyrosines. The relatively high level of VP1 in A185 was due to the high level of empty capsids in the A185 virus stock that entered the cells and persisted during infection.

Complementation of A185 genome accumulation and gene expression by wild type and mutant MT

In polyomavirus infected cells, both WTA2 and all the tyrosine mutant viruses also express ST. In order to study only the effect of MT, I expressed different tyrosine mutant MTs in NIH3T3 cells by retrovirus infection, followed by A185 infection as described in the Materials and Methods. DNA and protein samples were collected and analyzed by Southern blotting and Western blotting. The ability of different tyrosine mutant MT to complement the defect of A185 in genome accumulation and gene expression was assessed.

Figure 3-3A showed the levels of A185 genomes after 48 hrs of complementation by various MT mutants or control vector. The quantitation of the A185 genome increase is presented in Figure 3-3B. Compared to the control vector, WT MT increased the A185 genome levels by 6 fold; $Y3F_m$ by 4 fold and $Y3F_M$ MT by 1.5 fold, while the expression of Y6F MT failed to increase the A185 genome level. Y5F-250^+, Y5F-315^+ and Y5F-322^+ MT increased the A185 genome level by 2-2.5 fold, whereas Y4F-$250^+/315^+$, Y4F-$250^+/322^+$ and Y4F-$315^+/322^+$ MT increased the A185 genome level by 3.6-4.4 fold.

The expression of the various mutant MTs was high and relatively equal at 8 hrs (Figure 3-4A) and 48 hrs (Figure 3-4B) post A185 infection. Compared to control vector, the expression of WT and $Y3F_m$ MT increased the expression of A185 LT. But Y6F MT failed to do so. $Y3F_M$, quadruple mutants (Y4F) and quintuple mutants (Y5F) MT also increased LT expression to various degrees. A similar pattern was seen for VP1 expression, though the differences were small due to the high level of VP1 in A185 stock.

Both genome accumulation and protein expression results suggest that the six C-terminal tyrosines are involved in the MT regulation of both viral genome accumulation and gene expression. Among them, tyrosine Y250, Y315 and Y322 play a major role and tyrosine 258, 288 and 297 play only minor role.

The effect of MT on DNA replication could be either through LT or through transcription factors that bind to the polyoma enhancer or a combination of both. In order to separate these two effects, the effect of MT on A185 replication was compared in NIH3T3 and an NIH3T3-derived LT-expressing cell line. Cells were infected by retroviral vectors expressing WT or Y6F MT followed by A185. DNA and protein samples were analyzed by Southern and Western blotting (Figure 3-5A). Western blots confirmed the relatively equal levels of MT expression in these cells (Figure 3-5B). In NIH3T3 cells, the expression of WT MT increased the A185 genome levels by 50 fold (Figure 3-5A, left panel), while expression of Y6F MT led to a 5-fold increase. Thus, Y6F MT showed a 10-fold defect in the ability to complement the A185 replication defect. These results are similar to those shown in Figure 3-3A except that in that experiment the replication of A185 in the presence of Y6F MT was the same as that when there was no MT expression at all. This difference is likely due to the smaller scale of the overall genome accumulation in that experiment, since there was only 6 fold difference in A185 replication between with and without WT MT. I have observed a wide range of A185 replication defect, depending on a variety of factors, such as status of cells upon infection, batch of serum, etc. The small increase of A185 genome in the presence of Y6F MT suggests that MT functions other than those mediated by the six tyrosines may play a role in regulating viral genome accumulation.

Under conditions of high level LT expression in the NIH-LT cell line, the expression of WT and Y6F MT was still able to increase the level of A185 genomes by 5.6 and 2 fold, respectively (Figure 3-5B, right panel). Therefore, the effect of MT in A185 genome accumulation is not solely mediated through an increase in LT expression. Transcription factors activated by MT may also participate directly in DNA replication through polyoma enhancer.

MT activation of gene expression through polyoma enhancer

The ability of each tyrosine to activate the polyoma enhancer-dependent transcription was examined using a reporter gene assay. Retrovirus infection was carried out in EOL cells, an NIH3T3 cell line with an integrated -luciferase gene under the control of polyoma enhancer-origin sequence. Cell lysates were collected and were subjected to luciferase assay. As shows in Figure 3-6, the expression of wild type increased the luciferase activity by 3.7 fold. $Y3F_m$ MT was able to increase the luciferase activity to a level similar to that of WT MT. Removal of all three major tyrosines in $Y3F_M$ and Y6F MT reduced the luciferase activity to close to that of control vector. The presence of single major tyrosines caused only minor, if any, effects as shown in Y5F mutants. Y4F-250^+/315^+ and Y4F-250^+/322^+ MT increased luciferase activity by 2.4 and 1.8 fold, respectively. Interestingly, Y4F-315^+/322^+ MT activated the luciferase activity to close to that of wild type level. The significance of this finding remains to be determined.

DISCUSSION

The role of MT in polyomavirus replication has been demonstrated both in mice [12] and in cell culture [5, 6]. Possible mechanisms have been proposed: (1) MT can indirectly regulate viral DNA replication by increasing LT expression through the activation of polyoma early promoter; (2) MT can activate transcription factors (i.e. PEA1/AP-1 and PEA3/c-ets), which bind to the polyoma enhancer and stimulate DNA replication directly. Both processes are mediated by the activation of MT induced signaling pathways, which requires the phosphorylation of several MT C-terminal tyrosines. In this study, I investigate the role of six C-terminal tyrosines in viral genome accumulation and gene expression in cell culture. The following conclusions can be drawn from the results presented above: (1) Tyrosine 250, 315 and 322 play a major role among the six tyrosines being studied, while tyrosine 258, 288 and 297 all together play only a minor role. Each of the three major tyrosines contributes relatively equal to viral genome accumulation. (2) The six C-terminal tyrosines could not solely account for the defect caused by the loss of MT. (3) The effect of MT on DNA replication is attributed to an increase in LT expression as well as the activation of transcription factors that bind to polyoma enhancer and regulate DNA replication.

In this study, I used two approaches to examine the effect of MT tyrosine mutation on viral genome accumulation and gene expression. One approach used mutant viruses in a lytic infection, which more closely represents polyomavirus infection in nature. The other approach was to complement the MT-/ST- defective virus A185 with different mutant MTs by retrovirus infection. The MT complementation experiments eliminated the effect of ST on DNA replication, so that the role of MT in viral replication

and gene expression could be better isolated. To further separate the effect of MT on LT expression, MT complementation of A185 replication was carried out in a LT-expressing cell line. In the presence of abundant LT, MT was still able to increase the A185 genomes, though the magnitude was reduced (Figure 3-5B). This suggests the more direct role of MT on viral replication through transcription factors bound to polyoma enhancer.

Our results demonstrated that the function of C-terminal tyrosines in genome accumulation was parallel to their function in gene expression, both in terms of increasing early and late gene expression (Figure 3-4B) and activating polyoma enhancer-dependent transcription (Figure 3-6). However, in mutant polyomavirus infected cells, the differences of LT expression between WTA2, $Y3F_M$, $Y3F_m$, Y6F, MT-ter were small (Figure 3-2), while in the MT complementation experiment, the differences in LT levels were obvious (Figure 3-4B). One possible explanation is that WTA2, $Y3F_M$, $Y3F_m$, Y6F, MT-ter all expressed high levels of LT. Since LT can autoregulate its own synthesis, viruses expressing higher levels of LT will exert a stronger repression of its own LT synthesis, and therefore offsetting the MT effect on LT expression. A185 expresses very low levels of LT, so the promoting effect of MT on LT expression becomes distinguishable.

Tyrosine 250 is required [11, 15] and tyrosine 322 is dispensable [28] for cellular transformation. Tyrosine 315 is not essential for transformation, but mutation of tyrosine 315 to phenylalanine greatly reduces transformation efficiency [4, 17, 21]. In our study, the effect of each major tyrosine on viral genome accumulation and gene expression is similar, demonstrating a difference in the function of these tyrosines in viral replication and cellular transformation.

One interesting observation is that Y6F MT still displays partial capacity to promote genome accumulation and gene expression. Several possibilities can be speculated. (1) There may be binding sites for other proteins at the C-terminus of MT. For example, Serine 257 when phosphorylated, binds 14-3-3 [9, 22, 23]. 14-3-3 has been shown to bind PI3K [3], cdc25 [8] and Raf-1 [14]. 14-3-3 is involved in the Ras-dependent activation of Raf-1 [13, 26]. Mutation of the 14-3-3 binding site on MT does not affect MT transforming ability, but alters tumor profile [9]. (2) Other functions at the N-terminus of MT may also be involved. The DnaJ domain between residues 42 and 47 interacts with Hsc70 [24]. Hsc70 functions as a chaperone protein to promote the proper folding of proteins. This sequence is shared by all T antigens. It is possible that membrane-bound MT promotes the interaction of Hsc70 and its binding partner, which is involved in MT signaling. This interaction is abolished when MT does not bind to the cell membrane. The interaction between MT and PP2A is also essential for MT function. (3) The status of the viral minichromosome might be another contributing factor. hr-t mutants showed decreased level of histone H3 and H4 acetylation [27] compared to wild type virus, which implied a difference existed in chromatin structure. This difference may have strong impact on viral early gene expression. The 4- to 16-hrs delay of A185 in large T expression compared to wild type virus [5] provides evidence to support this hypothesis. The exact mechanism of how MT regulates viral replication is still under investigation. The functions proposed above may reflect the involvement of MT in various aspects of the polyomavirus lytic cycle, such as virus entry, early and late gene expression, viral DNA replication and virus encapsidation.

SUMMARY

In summary, this study demonstrates the role of C-terminal tyrosines in viral genome accumulation and gene expression and presents a possible mechanism for the role of MT in regulating DNA replication and gene expression. By activating different signaling pathways through these tyrosines, like it does for transformation, MT activates transcription factors (i.e. PEA1/AP-1 and PEA3/c-ets), which bind to the polyoma enhancer to either directly stimulate DNA replication, or indirectly through the activation of LT transcription. Although MT likely utilizes the same pathways for its functions in viral replication and transformation, the requirements for these pathways in the two processes are different. In transformation, the ShcA-Ras-MAPK pathway plays a more important role than the PI3K pathway, and the PLC-γ1 pathway is dispensable. In viral DNA replication, those three pathways contribute equally.

APPENDIX 3: FIGURES AND TABLES FOR CHAPTER 3

Table 3-1 Properties of polyomaviruses used in cell culture studies

This table lists the polyomaviruses used in this study and the properties of viral early proteins. WT = wild type; Mut = mutant; - = not expressed.

Notes:

a. X stands for the number of tyrosines that are mutated.

b. Single nucleotide (A to T) change in MT coding sequence does not affect LT amino acid sequence.

c. MT-ter expresses wild type ST as well as a truncated MT fragment which has the same amino acid sequence as ST, except that ST has 4 extra amino acids at the C-terminus.

Mutant	LT	MT	ST
A2	WT	WT	WT
YxF[a]	WT[b]	Mut	WT
MT-ter	WT	–	WT[c]
A185	WT	–	–

Figure 3-1 Comparison of viral genome levels in wild type and mutant polyomavirus infected cells

Cells were infected as described in Materials and Methods. Viral DNA was extracted at 6 and 48 hrs postinfection, digested with EcoRI, which linearizes the viral genome, processed for Southern blotting, and hybridized to a ^{32}P-labeled polyomavirus genomic probe as described in Materials and Methods. 1/2 of 6-hr DNA was loaded. 1/10 of 48-hr DNA from WTA2, Y3FM and Y3Fm and 1/3 of those from Y6F, MT-ter and A185 were loaded as shown. Hybridized counts were determined with a PhosphorImager and corrected for dilution, and the increase relative to the input is shown. The properties of each virus with respect to the expression of MT and ST are indicated. "+" = wild type; "M" = mutated; "-" = no expression.

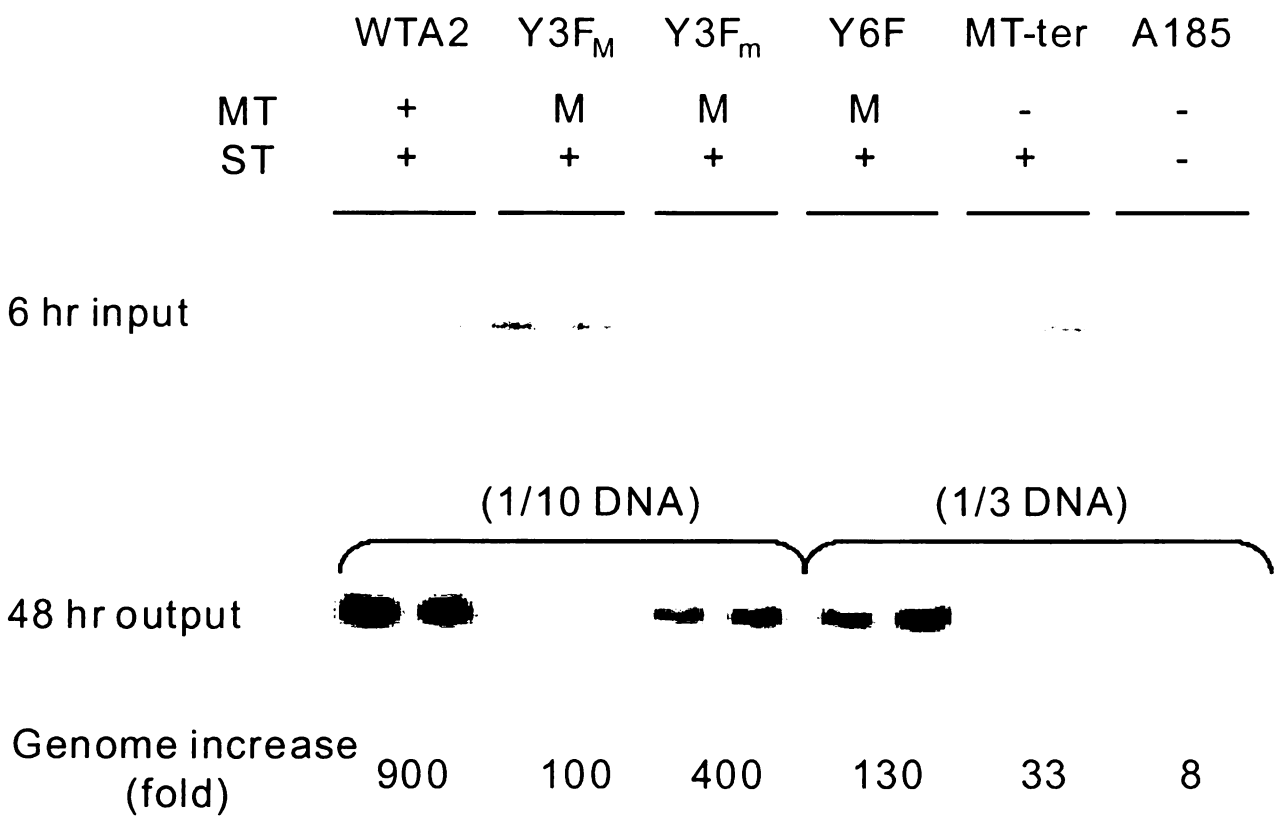

WTA2
Y3F$_M$
Y3F$_m$
Y6F
MT-ter
A185
MT + M M M - -
ST + + + + + -
6 hr input
(1/10 DNA)
(1/3 DNA)
48 hr output
Genome increase (fold)
900
100
400
130
33
8

Figure 3-2 Analysis of the early viral proteins

Cells were infected as described in Materials and Methods. Proteins were extracted at 48 hrs postinfection. (A) The Western blot was probed with the rat polyclonal anti-tumor serum. The three early polyomavirus proteins are identified on the left as large T (LT), middle T (MT), and small T (ST). This antiserum also detects two cellular proteins (identified with arrows), which serve as loading controls. (B) The blot shown in panel A was stripped and probed with a polyclonal rabbit anti-VP1 antibody.

Notes:

a. The level of VP1 in A185 infected cells is due to the entry and persistence of empty capsids in the A185 stock during infection.

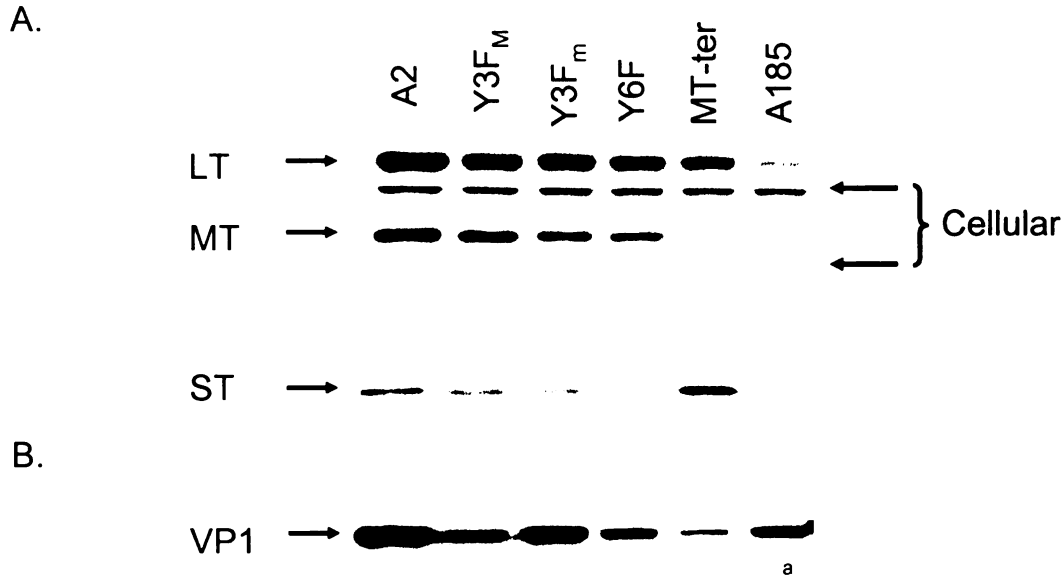
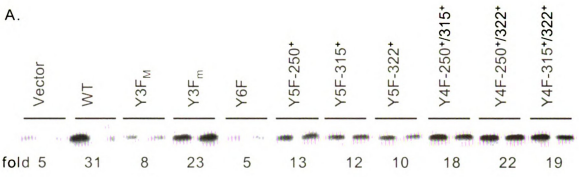

A.
A2
Y3F$_M$
Y3F$_m$
Y6F
MT-ter
A185
LT
MT
Cellular
ST
B.
VP1
a

Figure 3-3 Complementation of MTs in A185 genome accumulation

Cells were infected with retroviruses expressing different mutant MT for 20 hrs, then infected with A185 as described in Materials and Methods. DNA and proteins were extracted at 6 and 48 hrs post A185 infection. Duplicate samples were analyzed. (A) Southern blot was performed as described in the legend to Figure 3-2. The increase of A185 genomes is indicated. (B) A graphic presentation of the increase of A185 genomes in the presence of different mutant MTs, determined in the panel A. Duplicate samples are presented.

Note: Only one DNA sample of WT MT-expressing cells was analyzed.

A.


Vector
WT
Y3F$_M$
Y3F$_m$
Y6F
Y5F-250$^+$
Y5F-315$^+$
Y5F-322$^+$
Y4F-250$^+$/315$^+$
Y4F-250$^+$/322$^+$
Y4F-315$^+$/322$^+$
fold 5 31 8 23 5 13 12 10 18 22 19

B.

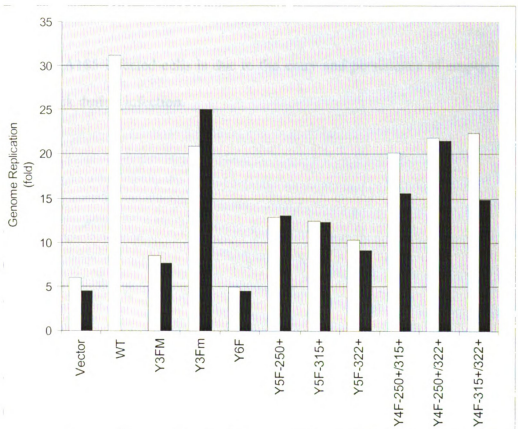

Genome Replication (fold)
35
30
25
20
15
10
5
0
Vector
WT
Y3FM
Y3Fm
Y6F
Y5F-250+
Y5F-315+
Y5F-322+
Y4F-250+/315+
Y4F-250+/322+
Y4F-315+/322+

Figure 3-4 Complementation of MTs on A185 viral early and late protein expression

Protein samples from the same experiment (as shown in Figure 3-3) were analyzed by Western blotting with the rat polyclonal anti-tumor serum for early and late protein expressions as described in the legend to Figure 3-2 (A) Protein samples at 6 hrs post A185 infection. MT is indicated on the left. Two cellular proteins are also detected. No LT is detected at this time. (B) Protein samples at 48hrs post A185 infection. LT and MT are indicated on the left. The blot was stripped and re-probed with a polyclonal rabbit anti-VP1 antibody as shown at the bottom.

Notes:

a. The level of VP1 in A185 infected cells is due to the entry and persistence of empty capsids in the A185 stock during infection.

A.


6hr input
Vector
WT
Y3F$_M$
Y3F$_m$
Y6F
Y5F-250$^+$
Y5F-315$^+$
Y5F-322$^+$
Y4F-250$^+$/315$^+$
Y4F-250$^+$/322$^+$
Y4F-315$^+$/322$^+$
LT
MT
Cellular

B.

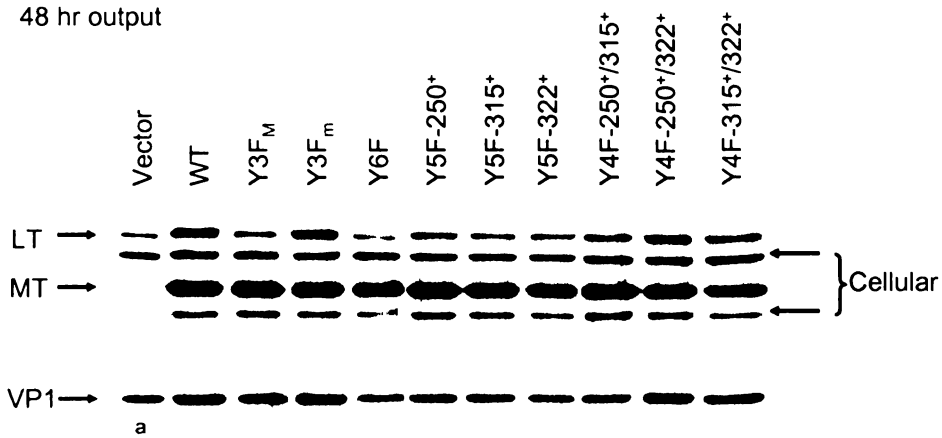
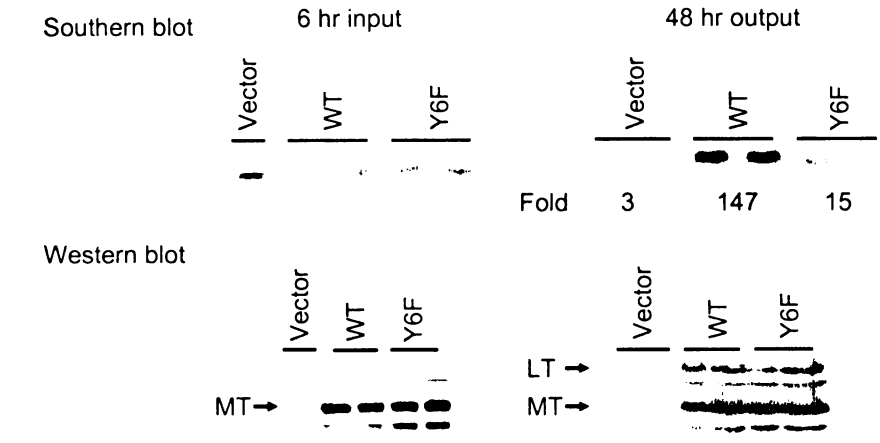

48 hr output
Vector
WT
Y3F$_M$
Y3F$_m$
Y6F
Y5F-250$^+$
Y5F-315$^+$
Y5F-322$^+$
Y4F-250$^+$/315$^+$
Y4F-250$^+$/322$^+$
Y4F-315$^+$/322$^+$
LT
MT
Cellular
VP1
a

Figure 3-5 Comparisons of MT complementation in NHI3T3 and NIH-LT cell lines

MT complementation of A185 genome accumulation was compared in NIH3T3 cells (A) and NIH-LT cell line (B). Cells were infected with retroviruses expressing WT and Y6F middle, then infected with A185 as described in the legend to Figure 3-3. DNA and proteins were extracted at 6 and 48 hrs post A185 infection and analyzed by Southern blot and Western blot as described in the legend to Figure 3-3 and Figure 3-4.

A. NIH3T3



B. LT cell line

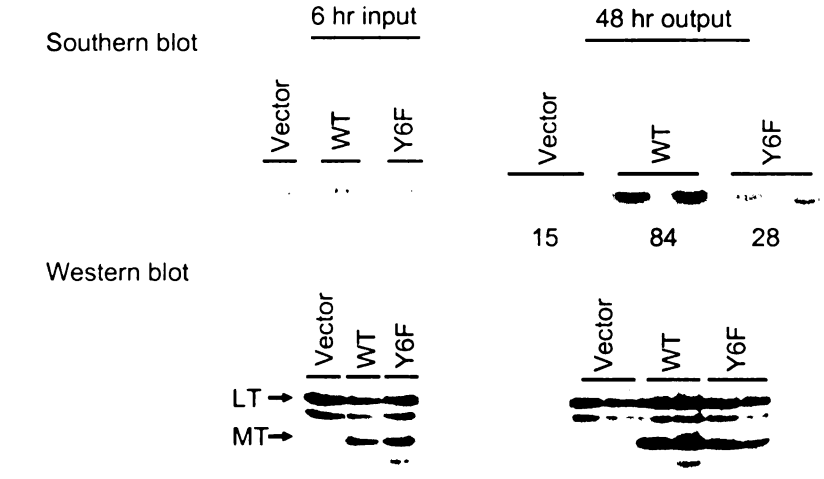


Figure 3-6 MT activation of gene expression through polyoma enhancer

NIH3T3 cells containing an integrated luciferase gene under the control of the polyoma enhance-origin (EOL) were infected with retroviruses as described in Materials and Methods. Luciferase activities were measured at 60 hrs postinfection and expressed as luciferase relative light unit with basal level (uninfected cells) set at 1. The increase in luciferase activity (average of duplicated measurements) is indicated above the column.

Luciferase Relative Light Unit
1400
1200
1000
800
600
400
200
0
Uninf.
Vector
WT
Y3FM
Y3Fm
Y6F
Y5F-250+
Y5F-315+
Y5F-322+
Y4F-250+/315+
Y4F-250+/322+
Y4F-315+/322+
1.0
1.0
3.7
1.2
3.6
1.2
1.6
1.6
1.4
2.4
1.8
3.6

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CHAPTER 4

THE ROLE OF MT C-TERMINAL TYROSINES IN GENOME ACCUMULATION AND PERSISTENCE IN MICE

ABSTRACT

In this study, I examine the function of middle T C-terminal tyrosines in viral replication in mice. A tyrosine sextuple mutant (Y6F) shows a 7-10 fold defect in viral genome accumulation in the acute infection of both neonatal and 4-week old mice. This defect becomes more severe in the 4-week persistent infection. The major tyrosine triple mutant ($Y3F_M$) shows a 2.4 fold defect, whereas the minor tyrosine triple mutant ($Y3F_m$) shows no defect in neonatal mice infection. Furthermore, a mild maturation defect is found in Y6F.

INTRODUCTION

MT has been shown to play important roles in viral DNA replication and gene expression in tissue culture [6, 7], as well as virus growth, persistence and tumor induction in mice [9, 16]. In the previous chapter, I demonstrated the role of six MT C-terminal tyrosines in viral genome accumulation and gene expression in cell culture. Here, I extend the study into the natural host of the virus -- the mouse. The interest was in comparing the function of middle T tyrosines in oncogenesis and in virus replication in mice. Mutant viruses were examined in both neonatal and 4-week young adult mice for possible defects in genome accumulation. Y6F was also examined for possible defects in

viral persistence and maturation. The results show that Y6F had a 7-10 fold defect in viral genome accumulation in both neonatal and 4-week old mice. This defect was more severe in persistent infection. In neonatal mouse acute infection, $Y3F_M$ showed only a 2.4 fold defect while no defect was found in $Y3F_m$. Moreover, a mild maturation defect was found in Y6F. These results demonstrate that the MT C-terminal tyrosines play important roles in viral replication in mice.

MATERIALS AND METHODS

Animal and polyomavirus infection

Balb/C mice at 4 weeks of age or in mid-pregnancy were purchased from the National Cancer Institute, Frederick. For neonatal mouse acute infection, mice were infected within 24 hrs of birth with $1x10^6$ PFU of each virus in 50 µl of 1X PBS subcutaneously in the dorsal region for 6 days. For young adult mouse acute infection, the same amount of virus was injected into 4-week old mice intraperitoneally.

Preparation and analysis of DNA

Mice were sacrificed and organs (skins, ribs, kidneys and lungs) were placed in digestion buffer (100 mM NaCl, 10 mM Tris [pH 8.0], 25 mM EDTA [pH 8.0], 0.5% SDS) containing 100 µg of proteinase K per ml, homogenized for 10 sec at 30,000 rpm with a Tekmar-Tissumizer, and digested overnight in a 50oC shaking water bath. Digested tissues were extracted with phenol-chloroform. Total DNA was precipitated with the addition of 1/10 volume of 3M sodium acetate and equal volume of isopropanol at -20oC for 1 hr. DNA was collected by centrifugation at 4oC and 10,000 rpm (Sorvall

SA-600) for 30 min. DNA samples were dried under vacuum and resuspended in TE (10mM Tris [pH 8.0] and 1mM EDTA [ph 8.0]). DNA concentrations were measured by absorbance at 260 nm with a spectrophotometer. For each sample, 10 μg of total DNA was digested with EcoRI (for single virus infection) or EcoRI +NheI (for mixed infection) and electrophresed on 1% agarose gels. DNA was transferred to Hybond nylon membrane (Amersham Pharmacia Biotech), UV crosslinked (Stratagene Stratalinker; 1200 micro-joules) and the blots were hybridized to a 32P-radiolabeled polyomavirus genomic probe. For single virus infection, the blots were stripped and re-probed with a probe hybridizing to the immunoglobulin gene μj region.

The hybridization was carried out at 65oC for 24-48 hrs in 1X Denhardt's solution, 2X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA [pH 7.7]). The hybridized blots were washed under stringent conditions. The blots were exposed to phosphorescent screens and scanned with the Phosphorimager (Molecular Dynamics). The blots were then exposed to X-ray films (Amersham Pharmacia Biotech) for up to 3 weeks at -70oC with an intensifying screen to achieve darker exposures. Statistic analysis was carried out by ANOVA method using SAS software package.

Live virus production

The amount of virus in the organs was determined by plaque assay using NIH 3T3 cells. Organs were placed in digestion buffer (100 mM NaCl, 10 mM Tris, [pH 8.0], 25 mM EDTA, [pH 8.0]) and homogenized as described above. The homogenates were frozen and thawed 4-5 times and clarified by centrifugation. The supernatant was used to infect NIH3T3 cells at 80% confluence. The infection was carried out at 37oC for 1 hr. Infected cells were fed with medium containing 5% serum and 0.9% agar and incubated

for 7 days at 37oC. The plates were stained with neutral red. The plaques were picked and resuspended in 20 µl of ddH2O. Following incubation at 45oC for 30 minutes, agar was removed by centrifugation. 10 µl of supernatant was used as the template to amplify a fragment of viral genome (nucleotide 41-1215) by PCR. PCR products were digested with XhoI, which cut the A2-RE DNA into two fragments (555 and 619 bp), but does not cut Y6F DNA (1174 bp).

RESULTS

The role of MT C-terminal tyrosines in genome accumulation in neonatal mice acute infection

To examine the role of three major tyrosines Y250, Y315 and Y322 in polyomavirus replication in neonatal mice, tyrosine to phenylalanine mutants were used. These mutants also express small T protein. For comparison, a mutant named MT-ter (MT^-/ST^+) which expresses ST and a MT fragment that is almost identical to ST and a mutant that produces neither MT nor ST, A185 (MT^-/ST^-) were also included.

The accumulation of viral genomes was used to track viral replication. Balb/C neonatal mice were infected, sacrificed 6 days after infection and total DNA was analyzed by Southern blotting. The level of viral genomes was compared. Figure 4-1 shows viral genome accumulation in the skin close to the site of injection. In the case of all tyrosine mutants tested, single (Y1F), double (Y2F) and triple ($Y3F_M$) mutants, genome levels were comparable to that obtained with WTA2, indicating no detectable defect. In contrast, MT-ter showed a 14-fold and A185 a 30-fold reduction in viral

genome levels. Skin distal to the site of injection was also analyzed as well as other organs such as the bone (ribs), the kidney and the lung and the results are summarized in Table 4-2. Since results from single (Y1F) and double (Y2F) mutants were similar to that of $Y3F_M$, only the data for $Y3F_M$ is shown. Overall, the levels of viral genome were high in skin, bone (ribs) and moderately high in kidney. But the genome levels in lung were relatively low even after 3 weeks of exposure, therefore the results were not included in Table 2. The genome tissue distribution agrees with the results from a previous study that demonstrated organ- specific replication pattern of polyomavirus infection [26]. Tyrosine mutants did not show an alteration of this pattern.

As shown in Table 4-2, in all organs studied, the Py/uj ratio for $Y3F_M$ was within a 2-fold range of that obtained with wild type virus, indicating no severe defect. MT-ter showed a 14-50 fold reduction, demonstrating the requirement of MT for efficient viral replication in mice. A185 showed the strongest defect with a 30-100 fold reduction. A 2-fold advantage of MT-ter over A185 in viral genome accumulation suggests a small role for ST compared to that of MT.

Given the lack of a severe defect for $Y3F_M$ in genome accumulation, a sextuple mutant Y6F was tested, in which all 6 C-terminal tyrosines were mutated to phenylalanine. Furthermore, a new strategy was developed to avoid variations between or within litters, or other experimental variations such as leakage of the virus input drop during infection, DNA recovery or loading. The strategy involved the use of a mixture of mutant with a reference wild type and allows direct comparisons in a single animal. One possible drawback of this strategy is that the expression of wild type MT may complement the defect of the mutant MT protein if the two viruses infect the same cell.

However, the probability of complementation should remain low, as demonstrated in Figure 4-5. The reference wild type, called A2-RE, is derived from WTA2 and contains added restriction endonuclease sites (e.g. *Nhe*I, *Xho*I).

To determine the ratio of the two viruses in the input, NIH3T3 cells were infected with the virus mixture and DNA was harvested prior to the onset of DNA replication, digested with *Nhe*I or *Xho*I and the genome ratio was calculated from the Southern blots. (Figure 4-2, input). The virus mixture was used to infect five litters of Balb/C neonatal mice subcutaneously for a 6-day acute infection. Total DNA extracted from various organs was analyzed by Southern blot (Figure 4-2). Both viruses achieved disseminated infection in the organs examined. Following the 6-day acute infection, A2-RE genome levels were higher than those found for Y6F by 4 to 18 fold, indicating a defect of Y6F in viral genome accumulation. Substantial variations between animals were observed, despite the mixed infection strategy. However, the defect in Y6F was relatively consistent in all organs studied, indicating that the Y6F defect is not organ-specific. The smallest defect was found in the skin close to the site of injection (average ratio of 6.2) compared to distant skin (ratio = 9.8) and other distant organs (bone = 9.4) and kidney (ratio = 11.8). This is indicative of a potential defect in dissemination (see below).

To further examine the role of the C-terminal tyrosines, a side-by-side comparison of different mutants was carried out using the mixed infection strategy. In this experiment, $Y3F_M$, $Y3F_m$, Y6F, MT-ter as well as WTA2 were included. Balb/C neonatal mice were infected and total DNA from skin close to the site of injection was analyzed by Southern blotting. Figure 4-3A showed a skin (close) sample results from 4 individual mice of each mixed infection. The overall results are summarized in Figure 4-3B. Both Y6F and

MT-ter had 7.6-fold defect in viral genome accumulation compared to WTA2, $Y3F_m$ and $Y3F_M$ ($p<0.001$). $Y3F_M$ showed only a 2.4-fold defect ($p=0.08$), which was not found in single virus infection (Figure 4-1). No defect was found with $Y3F_m$.

The role of MT C-terminal tyrosines in viral genome accumulation in neonatal mice persistent infection

The persistence of viral infection following infection of neonatal mice was also examined. Mice from the same litters as those examined in the 6-day acute infection (and shown in Figure 4-2) were kept alive for 4 weeks. Total DNA from the same set of organs of individual mice was analyzed by Southern blotting (Figure 4-4). An up to 100-fold decrease in the Y6F genome was observed. In most of the samples, the Y6F genome was barely detectable. The A2-RE/Y6F ratio was larger in samples where overall higher levels of viral genomes were maintained. These results showed that the defect of Y6F becomes more dramatic when followed through even a short persistence phase.

The role of MT C-terminal tyrosines in viral genome accumulation in 4-week young adult mice

Since the replication potential of polyomavirus in mouse progressively decreases with increased age of the mouse, I tested if tyrosine mutations also caused defects in adult mice. Previous studies in our lab demonstrated that replication potential declines with the age of the mouse in certain organs (kidney, liver and lung) with a marked drop around 3 weeks of age. Therefore mice of 4 weeks of age were used for adult mouse infection.

Balb/C mice at 4 weeks of age were infected with WTA2, A185 or doubly infected with WTA2 and A185 and sacrificed at 3, 5 and 7 days postinfection. These times were chosen to determine an optimal period of infection prior to the onset of the antiviral immune response that would clear the virus; both single and mixed infections were used to examine the possibility of complementation.

Total DNA from ribs was digested with MspI and analyzed by Southern blotting (Figure 4-5). *Msp*I digests the polyoma genome into 8 fragments. Because A185 has a 71 nucleotide deletion in *Msp*I fragment 4, the fragment 4 could be used to track the replication of each virus. In both WTA2 single and WTA2+A185 mixed infection, maximal genome levels were obtained at 5 days postinfection and decreased at 7 days postinfection despite the variations between two individual mice. Therefore, a 5-day infection period was used in adult mice infection in the following experiments. Compared to WTA2, A185 showed a severe defect in viral genome accumulation (Figure 4-5). The level of A185 fragment 4 in WTA2/A185 mixed infection was only slightly higher (less than 2 fold) than that in A185 single infection, suggesting that mixed infection does not cause serious complications.

Following the established protocol for adult mice mixed infection, Y6F was tested for a defect in genome accumulation. An up to 12-fold A2-RE/Y6F ratio was observed in organs studied (Figure 4-6). This defect was similar to that observed in neonatal mice acute infection, therefore was not age-specific.

Genome analysis of live virus production of polyomavirus A2-RE and Y6F

Polyoma middle T-defective hr-t mutants are defective in VP1 phosphorylation, resulting in defective virion maturation [10, 11]. Differences in the Y6F /WT ratio in skin close versus distant to the injection suggested the possibility of a maturation defect. To further test this possibility, levels of live virus in skin samples from both neonatal and young adult mice in the A2-RE/Y6F acute infection were measured. To this end, a plaque assay was carried out in NIH3T3 cells using tissue lysates. DNA from the plaques was used as a template to amplify a fragment of the viral genome spanning the XhoI site in the A2-RE genome. Thus, the parental origin of the viral genome in each plaque was determined (Figure 4-7).

In neonatal mice, 110 plaques were analyzed, among which 102 contained the A2-RE genome and 8 contained the Y6F genome. In 4-week young adult mice, 65 plaques were analyzed, among which 59 the contained A2-RE genome and 4 contained the Y6F genome. The ratio of A2-RE to Y6F genome was 13:1 and 15:1 in neonatal and 4-week young adult mice, respectively. A2-RE infected cells usually form larger plaques at earlier times than Y6F infected cells. Larger A2-RE plaques sometimes merge with small Y6F plaques, which lead to the presence of both genomes in a single plaque (Figure 4-7, arrows). In such cases, the plaque was treated as two, one attributed each virus. These 13-15:1 plaque ratios represent the accurate ratio of parental genomes in live, infectious viral particles of A2-RE and Y6F in the infected mouse skin. These live virus ratios are about 2-fold higher than the viral genome ratios in skin close to the site of infection (Figure 4-2 and Figure 4-6), and suggest that the Y6F mutant displays a modest defect in virion assembly.

DISCUSSION

The purpose of encoding a potent oncoprotein MT during polyomavirus evolution would be to provide advantages for virus growth and propagation, instead of causing tumor in its host. However the role of MT on virus replication in its natural host hasn't been studied extensively. In Chapter 3 of this thesis, I demonstrated the role of six C-terminal tyrosines in viral genome accumulation and gene expression in cell culture. Here, I further examined the replication of different tyrosine mutants in their natural host, both neonatal and young adult mice. The following conclusions can be drawn from the results presented above: (1) The six C-terminal tyrosines are required for efficient viral genome accumulation in mice, at both the neonatal and adult stage. The role of the minor tyrosines is very evident. (2) As expected, the loss of six tyrosines causes more profound defect in virus persistence than in acute infection. (3) The signaling events induced by these six tyrosines may have a small effect on maturation. (4) The age- and organ-specific replication pattern is not affected by tyrosine mutations.

In mice infected as neonates, the peak in virus titer is reached at 7-10 days postinfection [20, 21], which coincides with the onset of the immune response. This period represents the acute phase of polyomavirus infection. At 2 weeks postinfection, the immune response clears most of the viruses and infected cells, resulting in a dramatic decline in virus titer. Therefore in our study, mice were sacrificed at 6 days and 4 weeks postinfection, the former representing the acute phase and the latter a rather short period (approx 2.5 weeks) of the persistent phase of infection. The age of the mice at the time of infection also has an impact on viral replication [26], characterized by a marked decline

in viral replication potential in mice infected around 3 weeks of age [27] Therefore 4-week old mice were chosen for adult mouse infection.

Early studies by Freund et. al. demonstrated the role of MT in viral replication and persistence in mice [7, 9]. This is further confirmed by our results showing the defect in A185 in genome accumulation. Removing all six tyrosines caused a similar defect in viral genome accumulation as MT-ter. These results not only demonstrate the important role of these tyrosines in viral genome accumulation, but also suggest that these tyrosines accounts for the overall function of MT in viral replication in mice. These results are inconsistent with the results from cell culture experiments presented in Chapter 3, which showed MT-ter had a stronger defect than Y6F. Although I cannot rule out the possibility that the variations in the mouse experiment masked the actual difference between Y6F and MT-ter, it is possible that the presence of different cell types in organs may lead to more complex regulation of viral replication in mice than in cell culture. The smaller defect of $Y3F_M$ in mice (2.4 fold) than in cell culture (9 fold) provides another example.

The exact degrees of defect among $Y3F_M$, $Y3F_m$ and Y6F are difficult to estimate, due to the inconsistent results from cell culture and mice experiments. The functions of the major and minor tyrosines are likely redundant instead of being distinct, with the major tyrosines being more important. In such case, removing the minor tyrosines does not cause a severe defect in viral replication. When the major tyrosines are removed, the minor tyrosines can still partially compensate for the function. It is only when all six tyrosines are removed that the defect becomes apparent, especially when the growth conditions for the viruses are more stringent, e.g., a low level of serum in cell culture. The defect in Y6F genome accumulation is more severe in the 4-week persistent infection

than in acute infection. This is probably due to the accumulation of a small defect over multiple cycles of infection, or the decreased replication potential in adult mice, which pose more stringent conditions for viral growth, or a combination of both.

One interesting finding is that mutation of the tyrosine Y250 and Y315 did not cause a severe defect in viral genome accumulation in mice. Since tyrosine Y250 [8, 14] and tyrosine Y315 are important for transformation [4, 17, 18], our results suggest that the mechanisms of MT for regulating viral replication and cellular transformation are different.

By studying mice infected at the neonatal or adult stage, an organ- and age-specific pattern of viral replication was discovered [26]. In kidney, liver and lung, viral replication was very high after infection of neonatal mice but very low after infection of adults, while in mammary gland, skin and bone, viral replication was very high in neonates and moderately high in adults. The results presented here confirmed the viral replication pattern in neonatal and adult infection. But the six tyrosine mutation does not alter the age- and organ-specific viral replication pattern. The polyoma enhancer plays an important role in determining the age- and organ-specific pattern of polyomavirus replication in the mouse [2, 26]. The B enhancer domain mediates polyomavirus replication in most organs, in particular in kidney, at the neonatal stage but is not required in those organs which can sustain replication in the adult, i.e., mammary gland, skin and bone. The A enhancer domain mediates polyomavirus replication in the adult stage. In the alpha core of the A domain, there are binding sites for transcription factors PEA1/AP-1, PEA2 and PEA3/c-ets [15, 19, 23-25]. PEA1/AP-1 and PEA3/c-ets are proposed to mediate the role of MT and ST on viral DNA replication through the polyoma enhancer

[7]. The organs that support high levels of viral replication and persistent infection in adults are also the organs that develop tumors [27], suggesting that sustained viral genome replication and/or gene expression are required for oncogenesis. The present results imply that these six tyrosines are not involved in the determination of the age- and organ-specific viral replication pattern. This pattern is more likely determined by host transcription factors present at different stages of mouse development.

Polyomavirus host range transforming (hr-t) mutants [3] encoding defective MT and ST have been shown to have defects in VP1 phosphorylation, which affects virion assembly [10, 11, 13]. Casein kinase II (CKII) is responsible for the phosphorylation of serine 66 on VP1 and is often activated by serum, epidermal growth factor, and insulin-like growth factor [1, 5, 12, 22]. Therefore, it is possible that MT mimics growth factors to activate CKII, resulting in VP1 phosphorylation. I examined the Y6F for defect in virion assembly by measuring the production of infectious virus particles in the infected skins from both neonatal and young adult mice. The higher ratios of A2-RE/Y6F in live virus production compared to those average ratios in genome levels suggests Y6F has mild defect in virus maturation. This result is also supported by the observation that the ratios of viral genome level in tissues distal from the site of injection are relatively higher than those close to the site of injection.

SUMMARY

In this study, I demonstrated the role of six MT C-terminal tyrosines in viral replication in mice, with Y6F having a large defect in genome accumulation following

acute and persistent infection as well as a mild defect in virus maturation. In future studies, it would be interesting to examine the two triple mutants $Y3F_M$ and $Y3F_m$ for genome accumulation following persistent infection of neonatal mice so that minor defects could be uncovered.

APPENDIX 4: FIGURES AND TABLES FOR CHAPTER 4

Table 4-1 Properties of polyomaviruses used in mouse studies

This table lists the polyomaviruses and the properties of viral early proteins. WT = wild type; Mut = mutant; - = not expressed.

Notes:

a. x stands for the number of tyrosines that are mutated.

b. Single nucleotide (A to T) change in MT coding sequence does not affect LT amino acid sequence.

c. MT-ter expresses wild type ST as well as a truncated MT fragment which has the same amino acid sequence as ST, except that ST has 4 extra amino acids at the C-terminus.

Mutant	LT	MT	ST
A2	WT	WT	WT
YxF[a]	WT[b]	Mut	WT
MT-ter	WT	–	WT[c]
A185	WT	–	–

Figure 4-1 Polyomavirus genome accumulation in the skin close to the site of injection in neonatal mice following a 6-day acute infection

One to three litters of neonatal mice were infected as described in Materials and Methods. After 6-day infection, skins (close to the site of injection) from each litter were pooled and processed as described in Materials and Methods. Total DNA (10 μg) was digested with *Eco*RI, processed for Southern blotting, and hybridized to a ^{32}P-labeled polyomavirus genomic probe. To normalize for loading, the blots were striped and re-probed with a probe hybridizing to the immunoglobulin gene μj region. The ratio of Py to uj counts was calculated and compared to wild type A2.

Y3Fa 2nd
Y3Fa 1st
Y315/322F 3rd
Y315/322F 2nd
Y315/322F 1st
Y250/322F 2nd
Y250/322F 1st
Y250/315F
Y322F
Y315F
Y250F 2nd
Y250F 1st
MT-ter
A185
A2
# of pup
Py
uj
5
4
6
2
5
5
4
6
4
5
5
3
4
4
3

Table 4-2 A summary of viral genome accumulation in organs from neonatal mice following a 6-day acute infection

Neonatal mice were infected and polyomavirus genomes were analyzed as described in the legend to Figure 4-1. The Py/uj ratio of A2 was normalized to 1 and the Py/uj ratios of other viruses were compared to A2.

Notes:

a. The viral genome in lungs was relatively low even after 3 weeks of exposure, therefore the result was not included.

b. Results from single (Y1F) and double (Y2F) mutants were similar to that of Y3FM, therefore only Y3FM data was shown.

c. When the level of genome was too low to be detected, the comparison was made against background and a "greater than" ratio was calculated.

Organ[a]		A2 Py/uj Ratio	A2 Normalization	A185	MT-ter	$Y3F_M$[b]
Skin	Close	45	1.0	0.03	0.07	1.5
	Distal	38	1.0	0.01	0.02	0.5
Rib		45	1.0	0.02	0.04	0.4
Kidney		3	1.0	<.13[c]	<.16	0.5

Figure 4-2 Genome accumulation of polyomaviruses A2-RE and Y6F in neonatal mice following a 6-day acute infection

Five litters of neonatal mice were infected with a mixture of A2-RE and Y6F as described in Materials and Methods. After 6-day infection, organs from each litter (2 pups per litter) were pooled and processed as described in Materials and Methods. Total DNA (10 μg) were digested with EcoRI and NheI, which cuts A2-RE genome into 2 fragments, processed for Southern blotting, and hybridized to a ^{32}P-labeled polyomavirus genomic probe. The ratio of A2-RE to Y6F genome was calculated.

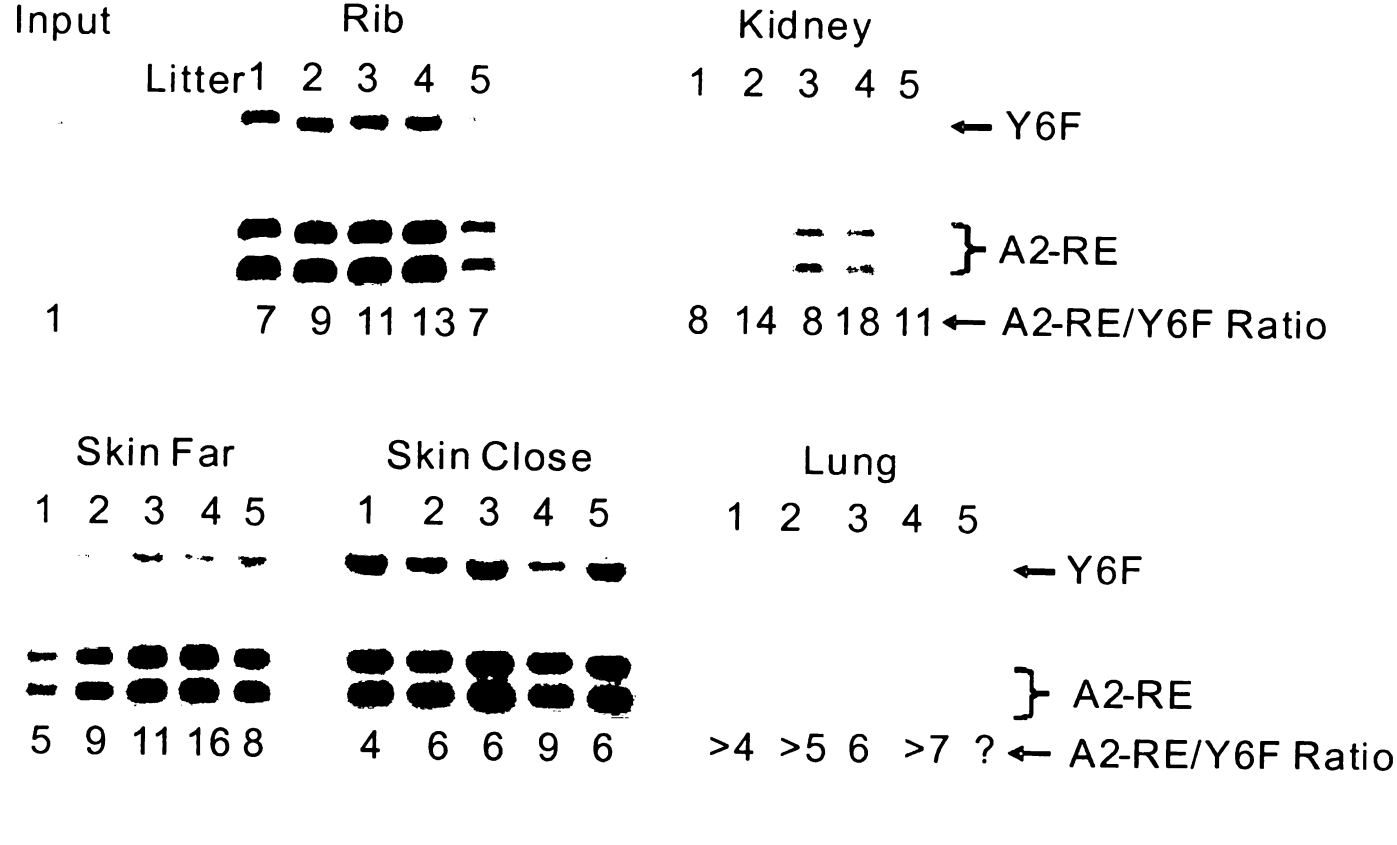

Input
Rib
Litter 1 2 3 4 5
1
7 9 11 13 7
Kidney
1 2 3 4 5
← Y6F
} A2-RE
8 14 8 18 11 ← A2-RE/Y6F Ratio
Skin Far
1 2 3 4 5
5 9 11 16 8
Skin Close
1 2 3 4 5
4 6 6 9 6
Lung
1 2 3 4 5
← Y6F
} A2-RE
>4 >5 6 >7 ? ← A2-RE/Y6F Ratio

Figure 4-3A Comparison of different mutants in neonatal mice following a 6-day acute infection

Neonatal mice were infected with a mixture of A2-RE and a distinct wild type or mutant virus as described in the Materials and Methods. At 6-day postinfection, skin (close to the site of injection) from individual mice was processed as described in Materials and Methods. Viral DNA was analyzed by Southern blotting as described in the legend to Figure 4-2. Results from five mice are shown. All blots were hybridized simultaneously using the same probe.

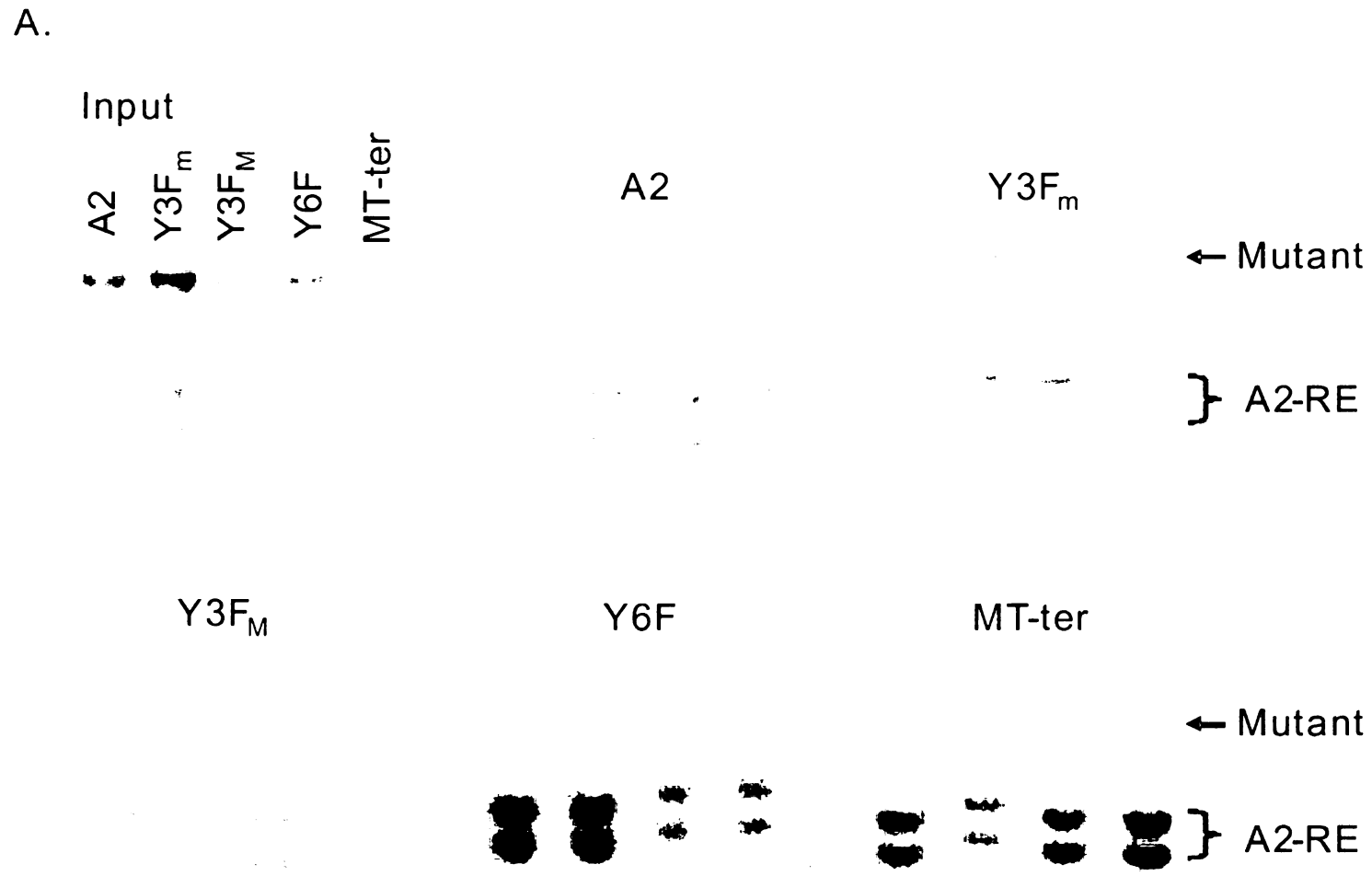

A.
Input
A2
Y3F$_m$
Y3F$_M$
Y6F
MT-ter
A2
Y3F$_m$
← Mutant
} A2-RE
Y3F$_M$
Y6F
MT-ter
← Mutant
} A2-RE

Figure 4-3B A summary of viral genome accumulation in skin (close to the site of injection) from neonatal mice following a mixed infection

This chart summarizes the defect of each mutant virus in genome accumulation described in Figure 4-3A. The results are the average of 8-16 mice in each group and the standard deviations is presented as error bars.

* $p < 0.001$

** $p = 0.08$

B.

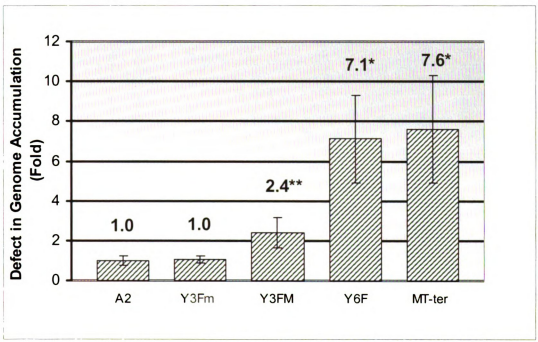

Defect in Genome Accumulation (Fold)
12
10
8
6
4
2
0
1.0
1.0
2.4**
7.1*
7.6*
A2
Y3Fm
Y3FM
Y6F
MT-ter

Figure 4-4 Genome accumulation of polyomaviruses A2-RE and Y6F in neonatal mice following a 4-week persistent infection

One or two neonatally infected mice from each litter (described in Figure 4-2) were kept alive for 4 weeks as described in Materials and Methods. Organ DNA from each mouse was analyzed by Southern blotting as described in the legend to Figure 4-2. The ratio of A2-RE to Y6F genome was calculated.

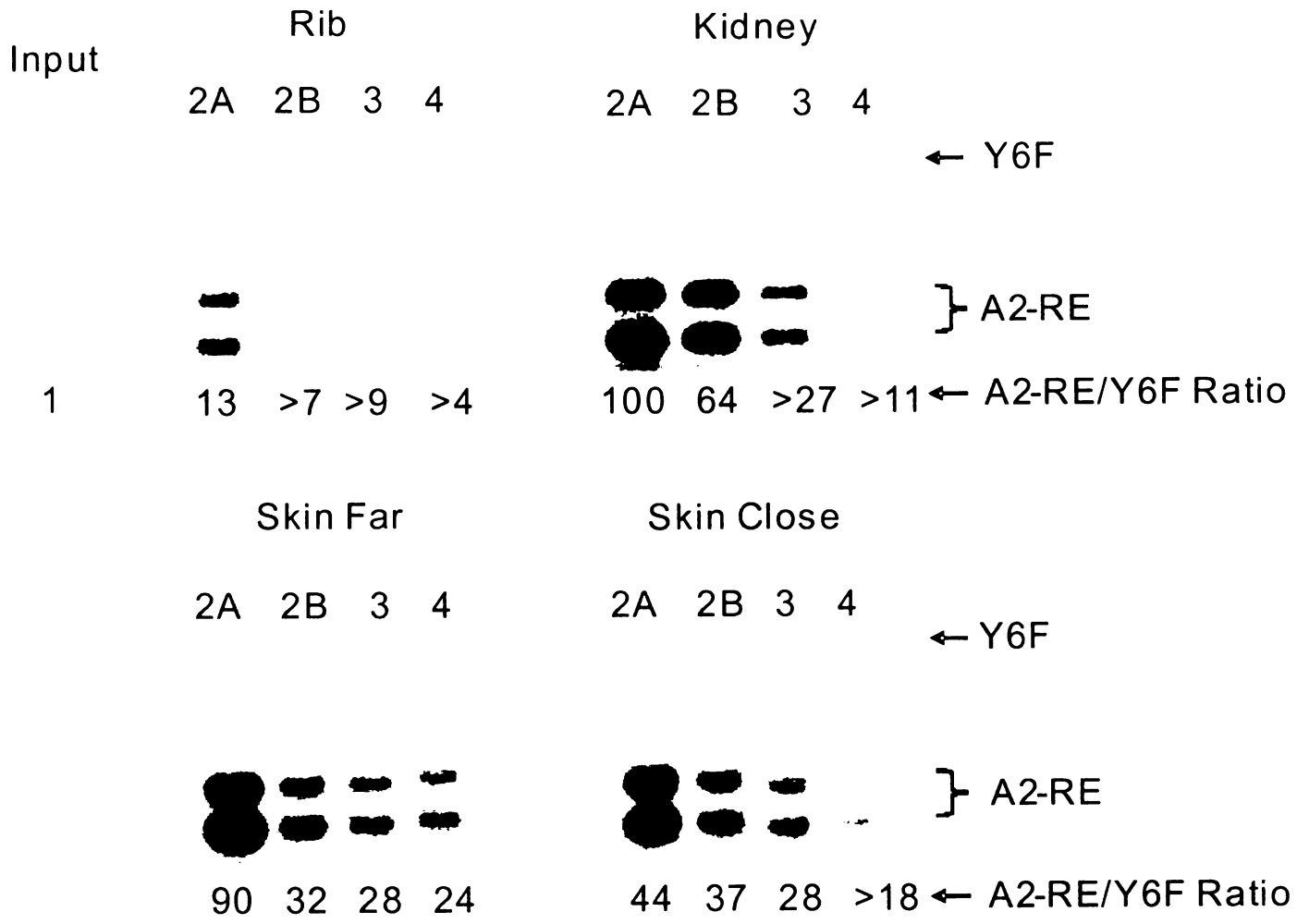

Rib
Kidney
Input
2A 2B 3 4
2A 2B 3 4
← Y6F
A2-RE
1
13 >7 >9 >4
100 64 >27 >11
← A2-RE/Y6F Ratio
Skin Far
Skin Close
2A 2B 3 4
2A 2B 3 4
← Y6F
A2-RE
90 32 28 24
44 37 28 >18
← A2-RE/Y6F Ratio

Figure 4-5 Determination of adult mouse infection period

4-week old mice were infected with A2, A185 or a mixture of both viruses as described in Materials and Methods. At the indicated time, Total DNA from ribs of two mice were digested with *Msp*I and analyzed by Southern blotting. The *Msp*I fragment 4 of each virus is indicated by arrows.

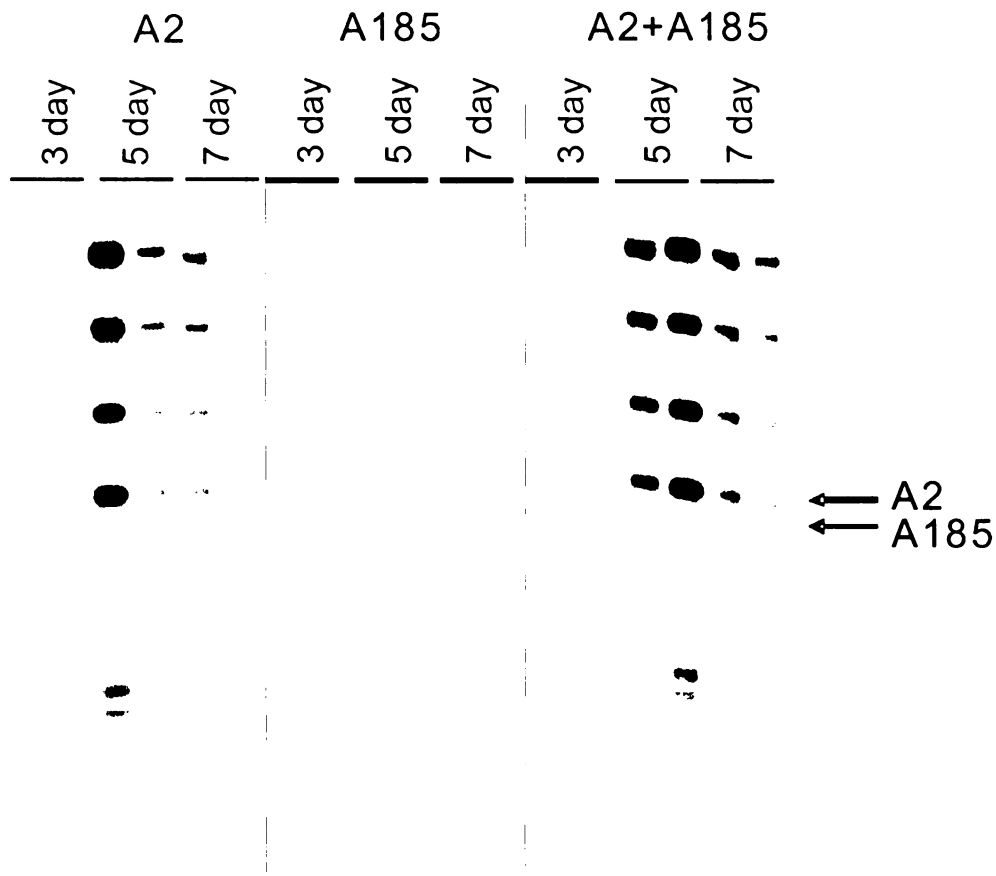

A2
A185
A2+A185
3 day
5 day
7 day
3 day
5 day
7 day
3 day
5 day
7 day
A2
A185

Figure 4-6 Genome accumulation of polyomaviruses A2-RE and Y6F in 4-week old mice following a 5-day acute infection

Three 4-week old mice were infected as described in Materials and Methods. Organ DNA was analyzed by Southern blotting as described in the legend to Figure 4-2 The ratio of A2-RE to Y6F genome was calculated.

* 3 week exposure

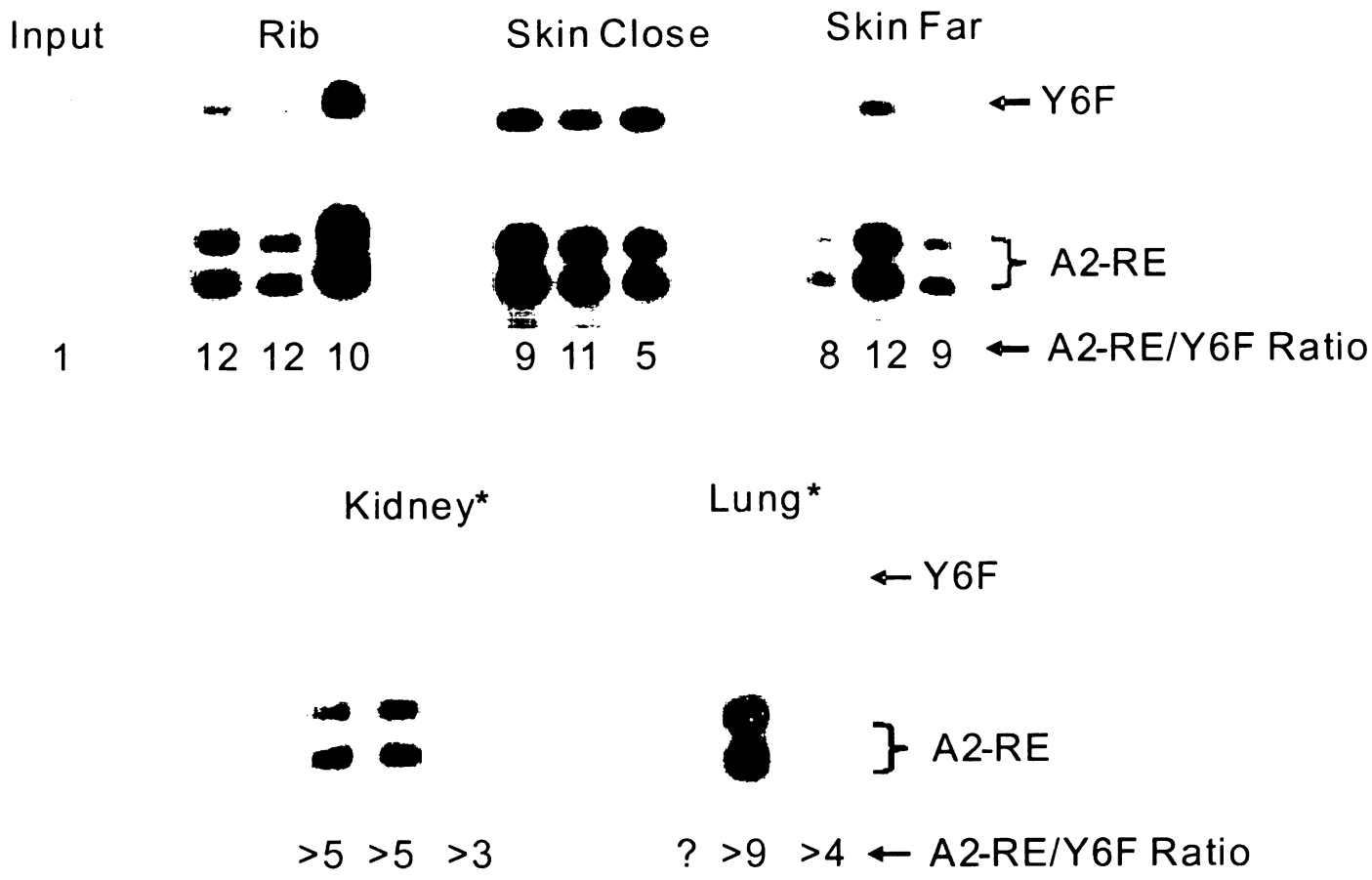
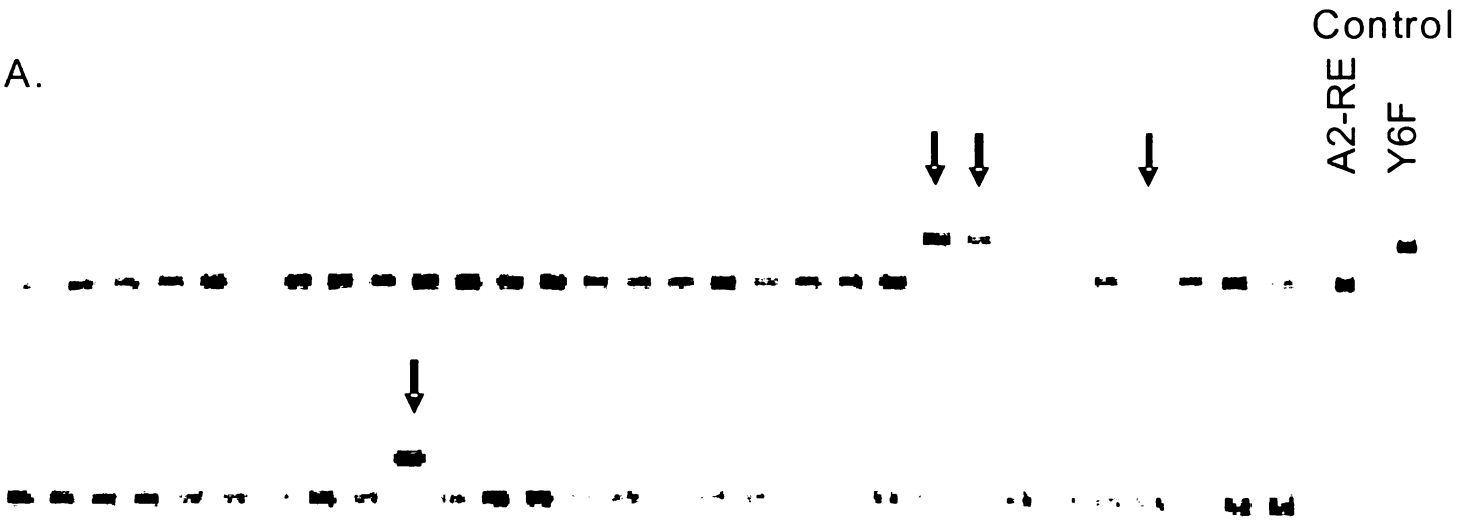

Input
Rib
Skin Close
Skin Far
← Y6F
} A2-RE
1
12 12 10
9 11 5
8 12 9
← A2-RE/Y6F Ratio
Kidney*
Lung*
← Y6F
} A2-RE
>5 >5 >3
? >9 >4
← A2-RE/Y6F Ratio

Figure 4-7 Genome analysis of live virus production of polyomavirus A2-RE and Y6F

Plaque assay was performed using organ homogenates. (A) Viral DNA from plaques were used as templates to amplify a fragment of viral genome spanning the XhoI site on A2-RE. *Xho*I digestion cuts A2-RE DNA into two fragments (555 and 619 bp), whereas Y6F DNA remains undigested (1174 bp). (B) Number of plaques containing each genome was counted.

A.



B.

Skin Sample(6day inf.)	A2-RE	Y6F	A2-RE/Y6F Ratio
Neonate	102	8	13:1
Adult	59	4	15:1

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