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A STUDY OF THE EFFECTS OF MIDDLE T AND SMALL T PROTEINS ON THE INDUCTION OF POLYOMAVIRUS DNA REPLICATION

by

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ABSTRACT

A STUDY OF THE EFFECTS OF MIDDLE T AND SMALL T PROTEINS ON THE INDUCTION OF POLYOMAVIRUS DNA REPLICATION

By

Cedric O. Buckley

Polyomavirus early proteins function both in viral DNA replication and transcription. In this study, the ability of middle T protein and small T protein to induce increased levels of polyomavirus DNA replication was investigated. Transient transfection assays using replication competent reporter plasmids containing the polyoma origin of replication indicate a 1.5 to 4.0 fold increase in the level of DNA replication when middle T protein was expressed. This replication induction requires *cis*-acting viral enhancer sequences. Small T effects on the induction of DNA replication were 1.5 fold or less.

A stable cell line containing integrated, replication competent plasmid DNA sequences was also used to test the effect of middle T and small T proteins on the induction of DNA replication. Qualitative analysis of data generated from these experiments further demonstrate that middle T protein can induce increased levels of DNA replication using a polyoma origin. The results obtained using both replication assay methods also suggest that the requirement

of the viral enhancer may reflect a direct role for certain transcriptional activators, through their ability to alter chromatin structure, as effector molecules for the regulation of DNA replication.

To my parents and my family.

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LITERATURE REVIEW

Introduction.

Murine polyomavirus is a member of the papovavirus family (1). The complete virion is approximately 45nM in diameter. The viral genome, a double-stranded, covalently closed circular DNA molecule of 5297 base pairs, is organized as a minichromosome complexed with cellular histones in an arrangement indistinguishable from that of cellular chromatin (2). It provides coding capacity for six viral proteins (figure 1.0). Three are regulatory in function and are expressed early after infection: large T (LT), middle T (mT) and small T (sT). The three remaining structural proteins viral protein 1 (VP1), viral protein 2 (VP2) and viral protein 3 (VP3) are expressed late in infection following viral DNA replication and oligomerize to form the viral icosahedral capsid. All other proteins required for viral replication and gene expression (e.g. RNA polymerase, DNA polymerase, accessory factors) are provided by the host (1,3). The genome also contains a non-coding intergenic region composed of sequences that provide viral regulatory functions. These sequences include: the

early and late transcriptional promoters, the origin of DNA replication and the enhancer. The enhancer contains binding sites for several cellular transcriptional activators and functions in the control of viral DNA replication as well as early and late transcription.

First isolated in 1953 (4), mouse polyomavirus was given its name [polyoma or "many tumors"] based on its ability to induce a wide variety of solid tumors in immunosuppressed mice and other rodents (5). The tumorigenic potential of polyomavirus has been studied extensively and has been genetically mapped to the early coding region of the viral genome. Specifically, large T is required for immortalization while the transforming ability of the virus is accomplished by middle T protein.

In permissive cells, polyomavirus undergoes a lytic infection resulting in viral replication and production of newly infectious virions. In cells from closely related species (e.g. hamster, rat), infection aborts with little or no genome replication and without production of infectious virions. Instead, a small number of viral genomes from these abortive infections can become stably integrated into the host genome. Abortive infection can result in neoplastic transformation of cells in tissue culture and tumorigenesis in susceptible animal models.

These features: a small, extensively-studied genome, packaging of the genome into a minichromosome, similar to the organization of cellular chromatin, and extensive use of cellular proteins throughout the viral life cycle make polyomavirus a model system well-suited to studies aimed at understanding processes involved in regulation of eukaryotic DNA replication. In this thesis, I have examined the role of the early viral proteins middle T and small T in the regulation of enhancer-dependent polyomavirus DNA replication. I present evidence suggesting that DNA replication is increased in the presence of middle T protein, while small T protein has little ability to cause increased DNA replication using a transient plasmid replication assay. Further, I present evidence that suggests a more pronounced role for middle T protein when DNA replication is measured using a cell line containing a stably integrated plasmid reporter. Since this DNA is organized into chromatin, one implication from these findings is the requirement for host reorganization of nucleosomal DNA templates that would promote efficient induction of DNA replication by middle T protein.

Strategy for polyomavirus DNA replication.

Polyomavirus contains a single, well-defined origin of bi-directional replication (figure 1.1) (6). This region contains the classical hallmarks of origins including: a 32 base pair G:C-rich palindrome, an A:T tract and three binding sites for the replication initiator large T protein (7). The A:T tract is the site for initial melting of the two DNA strands by LT during replication. The G:C-rich palindrome shares homology with similar sequences in the SV40 origin and contains sequences important for replication including a low-affinity large T protein binding site (6-8).

Large T protein is required for the initiation of viral DNA replication. It binds to both high and low affinity sequences within the origin region inducing localized melting and has ATPase/helicase activity that unwinds the two DNA strands. Replication initiation occurs in this "initiation zone" through the recruitment of RPA (the eukaryotic single-strand binding protein) and DNA polymerase-α/primase by large T protein and the assembly of the replication complex. Bi-directional replication occurs following RNA-primed synthesis of the first nascent DNA chain. Elongation is achieved through the activity of the

DNA polymerase-δ (or ε) complex resulting in the subsequent synthesis of new viral genomes (6,9).

Cellular signaling and transcription factor activation induced by middle T protein.

Through its hydrophobic, carboxy-terminal tail region, middle T protein inserts itself into the plasma membrane of expressing cells. There, middle T protein forms a stable complex with members of the c-src family of protein kinases including c-src, c-fyn and c-yes. The cellular response from the association of $pp60^{c\text{-}src}$ and middle T protein has been the most extensively studied. Binding of mT to c-src activates the tyrosine phosphorylation activity of c-src. Autophosphorylation of c-src occurs along with phosphorylation of middle T protein at key tyrosine residues (figure 1.2). Phosphorylation at tyrosine 315 of middle T protein causes association of the 80 kilodalton subunit of PI3-kinase with the activated mT/c-src complex. This, in turn, activates the enzyme activity of PI3-kinase resulting in increased production of D3 phosphatidylinositides. These lipids have been implicated in many cellular events including: cell motility, receptor trafficking and cell growth. One down-stream target of middle T-activated PI3-kinase is protein kinase B/Akt

(PKB/Akt) (10). The importance of this activation is not yet fully understood. However, activation of PKB/Akt has been shown to block apoptosis and promote mitogenic signaling in response to growth stimulation. This suggests a role for the PI3-kinase signaling pathway in cell growth regulation.

Phosphorylation at tyrosine 250 of middle T protein leads to binding of shc protein. This binding, along with that of adaptor molecules sos and grb2, couples upstream receptor signaling to a phosphorylation cascade involving ras and raf as well as MEK and MAP kinase. This signaling cascade activates nuclear factors that can, in turn, regulate transcription and replication upon binding to their respective recognition sequences within DNA (11,12).

Phosphorylation at tyrosine 322 of middle T protein leads to phospholipase C-gamma binding to the mT/c-src complex. Activation of this phosphodiesterase leads to the cleavage of membrane-bound, cellular phophatidylinositol-4,5-bisphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol. Diacylglycerol is a potent activator of protein kinase C, PKC (13). PKC, a serine/threonine kinase, directly activates raf through phosphorylation. At this point, signaling continues as with the tyrosine 250 pathway with one result being activation of nuclear transcription

factors. Thus two signaling pathways, one activated through mT-pp60 $^{c\text{-}src}$/shc and the other through mT-pp60 $^{c\text{-}src}$/PLC-gamma, converge at the point of raf activation.

One clear end result of this cascade is the activation of several nuclear transactivators including AP1/PEA1 and c-ets/PEA3 (11,12). Activation of AP1/PEA1 by middle T protein was demonstrated first using a middle T-transformed rat fibroblast cell line. A beta-globin gene transcription reporter construct containing four tandem copies of the AP1/PEA1 DNA binding sequence or a control vector with no AP1/PEA1 DNA binding sequences was transfected into these cells. Analysis of total RNA for initiation from the beta-globin promoter using S1 nuclease mapping revealed that the middle T-transformed cell line efficiently activated transcription when AP1/PEA1 DNA binding sequences were present in the reporter plasmid. Subsequent experiments using a short-term transfection protocol confirmed that upon expression of middle T protein, transcription was activated from the AP1/PEA1-responsive reporter plasmid and not from a plasmid with a mutated DNA binding sequence (14). In similarly designed experiments, the c-ets/PEA3 transcriptional activator was also shown to be activated upon expression of middle T protein (15).

Enhancers, transcriptional activators and their effect on polyomavirus DNA replication.

Enhancer elements are comprised of cis-acting, DNA sequences first discovered and characterized in polyomavirus and SV40 non-coding regions, and the sea urchin H2A gene (16,17). They are broadly defined by their ability to activate gene expression when linked to a variety of transcriptional promoters. General features of enhancers include: relatively short sequences, ability to function independently of orientation, and ability to exert regulatory effects over relatively long distances.

Efficient polyomavirus early transcription requires the enhancer region (18). Enhancers have also been shown to augment DNA replication of recombinant plasmids containing the SV40 origin of replication (19). Several previous reports (and results presented in this study) established that replication initiating from the polyomavirus origin also requires the enhancer. One of the earliest studies demonstrating this *cis*-effect of the enhancer region focused on the replication of polyomavirus deletion mutants. Since many of the mutants also had defects in early gene expression, helper virus expressing early viral proteins was co-transfected with the mutant genomes. Alternatively, deletion mutants were transfected into a

cell line (COP-5 line) constitutively expressing polyomavirus early proteins. Replication was measured using a loss of DNA methylation-based assay similar to the strategy used in the present study. Several mutant genomes were unable to replicate and the deletions that conferred this defect in replication were mapped to the enhancer region (18). In a subsequent report, the polyomavirus enhancer was replaced by analogous sequences from the simian virus 40 (SV40) regulatory region to determine if the activation of DNA replication was an inherent property of enhancers. Results showed that SV40 enhancer sequences could function to activate DNA replication using a polyomavirus origin. In addition, replacement of the polyomavirus enhancer by a cellular enhancer from the mouse immunoglobulin heavy-chain gene (which functions in lymphoid but not fibroblast cells) was able to activate polyomavirus DNA replication in a myeloma cell line but not in the COP-5 cell line. This provided evidence suggesting that the ability to activate DNA replication was a general feature of enhancers. These results, along with others, also established that enhancers could regulate replication in a cell-type specific manner (17,20).

Fine-mapping studies using extensive libraries of deletion mutants as well as discrete point mutants

dissected the enhancer into several functional domains (figure 1.3). The enhancer was divided into alpha-and beta-enhancer domains, each of which can function independently (and with some redundancy in activity) for both DNA replication and gene expression (21-26). Each of these enhancer domains is comprised of both core and auxiliary sequences (24). The beta-core is active alone as an enhancer domain, but can be augmented by beta-auxiliary domains 1 and 2 which are located on either side of the core. The beta-core/auxiliary domain contains sequences that are 80% homologous to SV40 core enhancer and provides binding sites for several cellular transcriptional activators in vitro (27,28). The alpha-auxiliary domain, which contains an A:T rich stretch, (nt 5073-5102) can augment alpha-core enhancer activity, but no cellular factors have been shown to bind this region to date. The alpha-core (nt 5108-5126) includes a sequence that is perfectly homologous to adenovirus E1A enhancer core (24) with a complete PEA3 (mouse homolog to human c-ets) binding site, as well as PEA1 (mouse homolog to AP1) and PEA2 binding sites (29,30).

In order to identify more precisely which binding sites affected the functioning of these domains, viral genomes containing point mutations within the enhancer were

characterized both for transcription and replication function. Several mutant viral genomes defective in replication and transcription were shown to have alterations in a region of the alpha-core enhancer spanning the binding site for the AP1 transactivator protein. Revertant genomes were also isolated and characterized. Reversions that restored the AP1 binding site restored replication and transcription to nearly wild-type levels (31). In another study, oligonucleotides containing either the AP1 binding site, the c-ets binding site or mutated versions of each were synthesized and inserted into test plasmids to measure the effect that AP1 or c-ets transactivator binding would have on DNA replication and transcription. Using transient plasmid replication assays and measuring gene expression using the chloramphenicol acetyltransferase reporter, it was demonstrated that: *i*) either AP1 or c-ets could activate DNA replication and transcription, *ii*) AP1 and c-ets could act synergistically to activate DNA replication, *iii*) the AP1 binding site was indispensable for the functioning of the α-core enhancer (32).

The ability of components of the AP1 complex to modulate DNA replication was also investigated. AP1 is a dimeric transcriptional activator protein consisting of

members of the *jun* and *fos* gene families. The protein can exist as a heterodimer of c-jun and c-fos or a homodimer of two c-jun molecules. Both members of the AP1 complex have transcriptional activation domains. However, only the *jun* gene family members have sequence-specific DNA binding activity. To test the ability of a single activation domain to regulate DNA replication, chimeric proteins were constructed containing a surrogate DNA binding domain from the *E. coli* lex A-repressor fused to the transcriptional activation domain of either c-jun or v-jun (a constitutively active and, therefore, oncogenic version of c-jun). Replication test plasmids were constructed containing lex A-operator sequences and plasmid replication levels were measured following transfection and expression of the chimeric proteins. Both c-jun and v-jun were able to activate DNA replication so long as the activation domains were intact (33). From this accumulated evidence, the transcriptional activators AP1/PEA1 and c-ets/PEA3 (and their corresponding DNA binding sites), were shown to be crucial for activation of enhancer-dependent DNA replication and alpha-core transcriptional enhancer activity.

Mechanisms of transactivator function and the role of middle T protein in the regulation of DNA replication.

Increasingly, transcriptional activators have been identified as having a significant role in regulating DNA replication (34-38). Transactivators can utilize at least three mechanisms to achieve this regulation. These mechanisms are not necessarily mutually exclusive.

First, bound transactivator proteins can facilitate assembly of the replication complex either directly by interacting with proteins of the replication complex or indirectly by altering the chromatin structure of adjacent regions of DNA making them more readily accessible to the replication complex. Evidence of a requirement for chromatin remodeling in the activation of DNA replication is somewhat indirect. In one report, the polyomavirus enhancer was integrated into a human fibrosarcoma cell line at a single-copy genomic locus for the HPRT gene. No endogenous enhancer was contained within this gene and DNase I sensitivity was low prior to targeted gene disruption which aided in subsequent analysis. Results from this study indicate that the polyomavirus enhancer can mediate the formation of a region of chromatin accessibility centered at the enhancer and extending outward in both directions over several kilobases of DNA

(39). Experimental data that support chromatin remodeling as a mechanism for activation of DNA replication comes from in vitro replication studies using the polyomavirus origin. Replication of polyoma origin-containing DNA templates in a cell-free system occurs efficiently in the absence of the polyomavirus enhancer (40,41). This contrasts with the requirement for an enhancer to obtain replication in vivo. Since the templates used in the in vitro studies are not assembled into chromatin, the results suggest that the enhancer and the transactivators that bind within it are required to modify chromatin structure.

Secondly, transactivators may direct DNA templates to subcellular compartments called nuclear replication foci. There is an accumulating body of evidence that suggests that the foci are the sites within the nucleus where eukaryotic DNA replication occurs. These foci are attached to the nuclear matrix (also called the nuclear scaffold), which has been shown to co-localize with several members of the replication complex including: DNA polymerase α, the processivity factor PCNA, and the single-strand DNA binding protein RP-A (42). The nuclear factor PEBP2αB/PEA2 is related to nuclear matrix protein-2 (NMP-2), a member of the AML/CBF/runt domain transcription factor family (43). NMP-2 binds the same DNA sequence as PEA2 and is found

localized in the nuclear matrix. Results from one study showed that PEA2 alone can bind the polyomavirus enhancer and activate DNA replication using test plasmids containing a polyoma origin. A replication activation domain of PEA2 was mapped and shown to provide at least two different functions: targeting to the nuclear matrix and association with a DNA replication protein (44).

Finally, transactivators may interact with an assembled replication complex either directly or through cellular co-factors to regulate the activity of the complex. During initiation of polyomavirus DNA replication, large T protein melts and unwinds the DNA in the origin region (9). In two separate studies using similar techniques to measure in vitro DNA unwinding, AP1 and c-jun alone were shown to stimulate large T-dependent unwinding of DNA templates containing the AP1 binding sequence. The stimulation of DNA unwinding by c-jun was mapped (using truncation mutations) to the activation domain of the protein. Also, evidence for the stimulation of large T protein binding to the polyomavirus origin by the activation domain of c-jun was obtained using chemical cross-linking methods following gel mobility shift assays of in vitro binding reactions. The binding specificity of large T prctein and the stimulation of large T-origin

complex formation by c-jun were both confirmed by Dnase I footprinting analyis (45). Both studies also demonstrated a direct physical interaction between c-jun and large T protein (45,46). Taken together, these results show that c-jun activates replication directly by stimulating large T-origin complex formation and by increasing the ability of large T protein to catalyze ATP-dependent unwinding of origin sequences during initiation of replication.

Viral DNA replication experiments in our laboratory with mT/sT mutants identified a role for middle T protein in viral DNA replication (47). One experiment in that report measured the accumulation of genomes following viral infection of NIH3T3 cells cultured in low serum-containing media. A wild-type strain of polyomavirus (WTA2) was compared to a mutant virus derived from WTA2 (A185), which does not produce middle T or small T proteins. The results showed a 16-fold reduction in genome replication for A185 versus WTA2. This result suggests that the replication advantage of WTA2 is mediated by middle T/small T protein. In later experiments, the role of the enhancer in genome replication was explored. A recombinant virus [A185(enhancer-duplicated)] in which a duplicated enhancer fragment was used to replace the single enhancer in A185 was constructed and its replication in tissue culture was

compared to that of A185. The results showed that in low-serum conditions, [A185(enhancer-duplicated)] had a replication advantage compared to A185, suggesting a role for enhancer sequences in viral replication. If the replication advantage observed was due to competition for some limiting cellular factor(s) which could bind within the enhancer, then results from the experiments with middle T deficient viruses suggest that middle T is capable of increasing the availability of this cellular factor(s). This would have the effect of reducing the replication advantage of viruses with enhancer duplications. Cellular transcription factors activated by middle T protein (e.g., AP1, c-ets) are clearly candidate cellular proteins capable of mediating this type of regulation.

Although the above data strongly support a role for middle T protein, these experiments do not provide conclusive evidence that middle T protein alone is capable of activating polyomavirus DNA replication. Two difficulties arise when using viral infection experiments to study replication of the viral genome. The first is the ability to produce viruses defective in middle T expression but capable of expressing the remaining viral proteins. In the viral infection experiments discussed above, A185 did not express either middle T or small T protein. Secondly,

middle T protein signaling activates cellular phosphorylation events resulting in the modification of capsid proteins in the virion. The absence of this capsid modification when studying middle T mutants has the potential to affect infection efficiency and cause variations in replication that are not the result of middle T protein effects on genome replication. A further difficulty arises from the fact that all of the early proteins of polyomavirus are encoded by sequences in the viral genomic DNA that overlap extensively. Large T, middle T, and small T proteins are translated using three mRNAs produced by differential splicing of one primary RNA transcript. All three mRNAs share common 5′ and 3′ regions including a transcriptional early promoter. Transcription from this promoter is activated by middle T protein in the presence of the viral enhancer. The result is that in a productive viral infection, expression levels of the early proteins are auto-regulated both quantitatively and temporally.

The current study attempts to avoid these pitfalls by measuring replication using an assay based on the co-transfection of cells in tissue culture with both middle T and large T protein expression plasmids along with reporter plasmids containing the polyomavirus origin of replication linked to polyomavirus enhancer sequences.

APPENDIX A

Chapter 1 Figures

Figure 1.0. Physical map of the polyomavirus genome. The inner circle represents the MspI/HpaII restriction map of the viral genome. The numbers 0-100 within the inner circle refer to physical map units while 0-5292 on the outside of the inner circle are nucleotide numbers. The early and late protein coding regions are depicted by the outer circles. Each region produces a unique mRNA coding for either T antigens (large T, middle T, small T) or capsid proteins (VP1, VP2, VP3) from a common RNA precursor by differential splicing (jagged lines represent the introns). The non-coding regulatory region (dashed lines) contains the viral origin and enhancer and is located between nucleotides 5021-5265. This figure is taken from (Soeda *et. al.*, 1980).

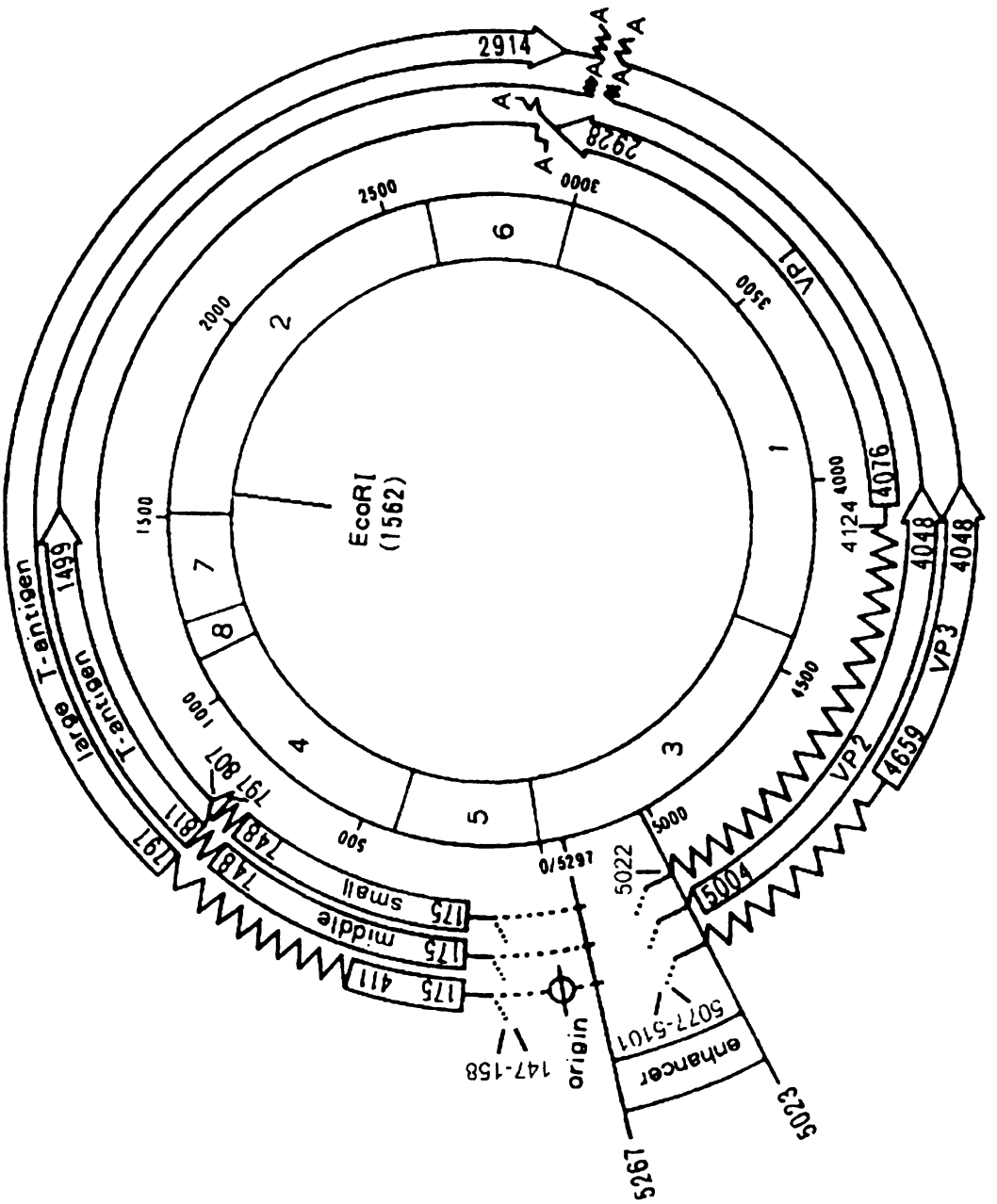

2914
2928
VP1
4076
4048
4048
VP3
4659
VP2
5004
5022
5077-5101
enhancer
5023
5267
origin
147-158
175 small
175 middle
175 411
748
791
T-antigen
large T-antigen
1499
EcoRI
(1562)
1
2
3
4
5
6
7
8
500
1000
1500
2000
2500
3000
3500
4000
4500
5000

Figure 1.1. Physical map of the 244 base pair enhancer region of the polyomavirus genome. The enhancer has early and late boundaries at restriction sites PvuII (nucleotide 5267) and BclI (nucleotide 5023) respectively. Transactivator binding sites are labeled. The alpha domain (A) is located between restriction sites BclI (nucleotide 5023) and PvuII (nucleotide 5130). The beta domain (B) is located between restriction sites PvuII (nucleotide 5130) and PvuII (nucleotide 5267). The alpha and beta core regions are designated below the diagram. (ORI) is the origin of replication. (L) and (E) are the late and early transcriptional start sites.

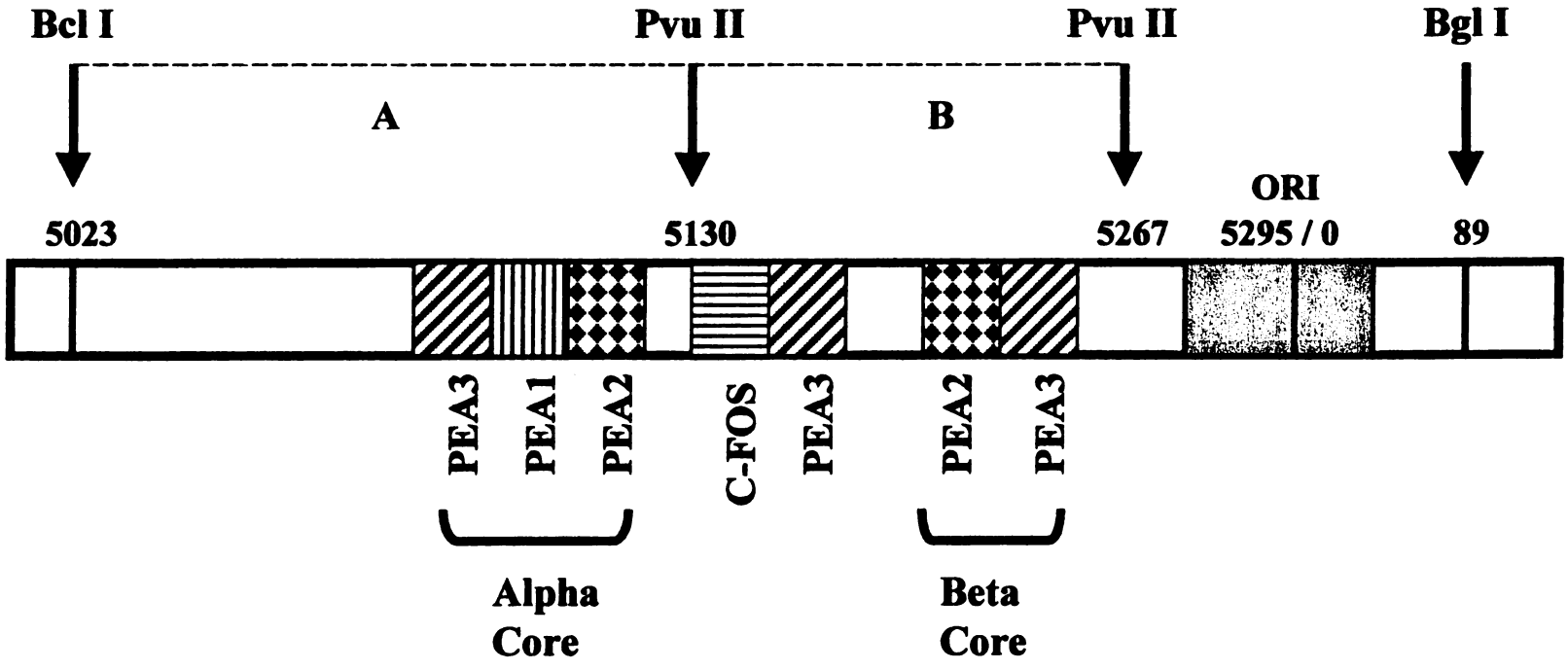
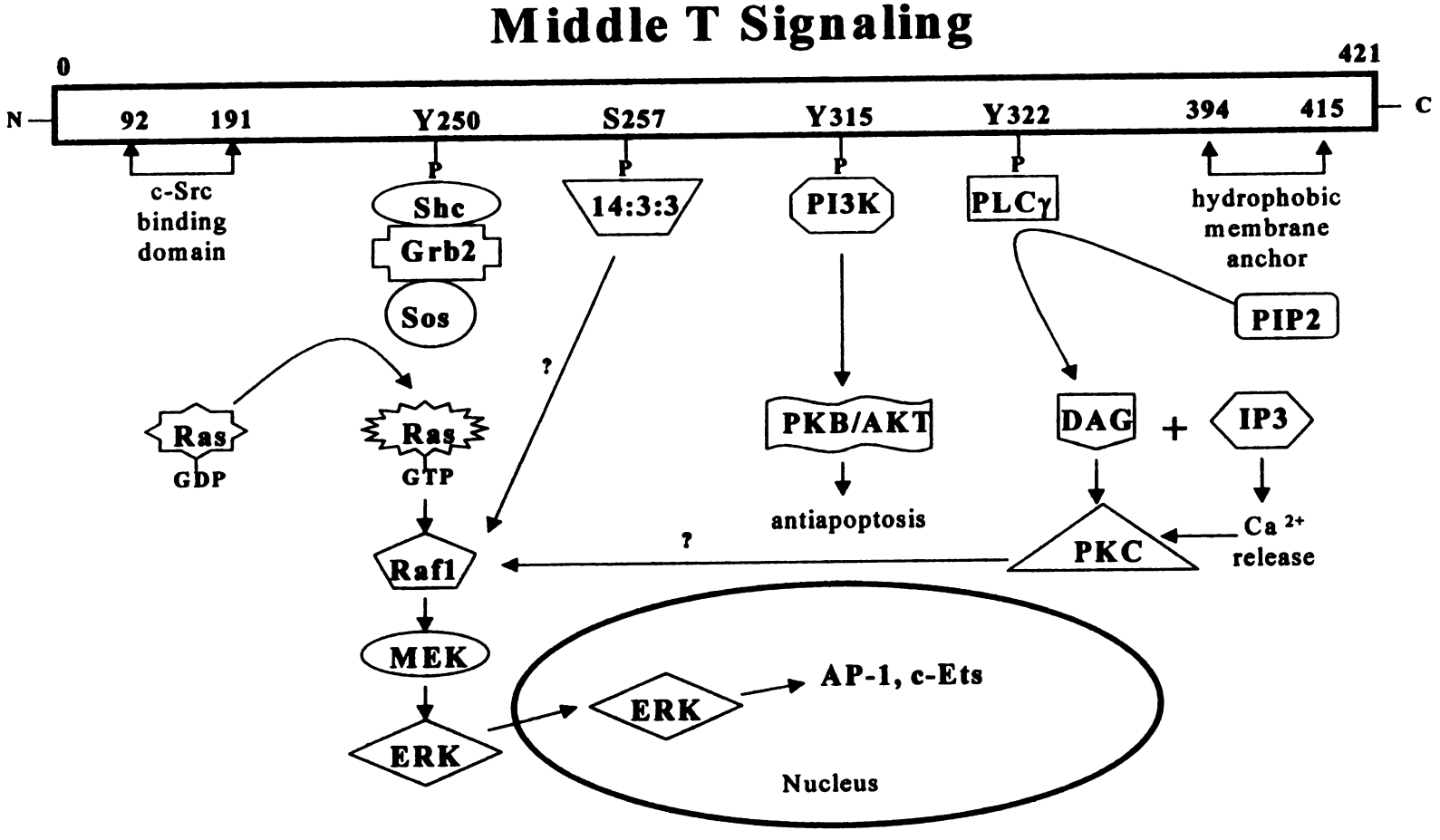

Bcl I
Pvu II
Pvu II
Bgl I
A
B
ORI
5023
5130
5267
5295 / 0
89
PEA3
PEA1
PEA2
C-FOS
PEA3
PEA2
PEA3
Alpha
Core
Beta
Core

Figure 1.2. Intracellular signaling cascades activated by middle T protein/c-src complex. The top portion of the figure represents the primary amino acid sequence of middle T protein. The three major signaling cascades are activated as a result of phosphorylation events at tyrosine residues 250, 315 and 322. The bottom portion of the figure represents several nuclear transcription factors activated through these signaling cascades.


Middle T Signaling
0
421
N
92
191
Y250
S257
Y315
Y322
394
415
C
c-Src binding domain
P
Shc
Grb2
Sos
14:3:3
PI3K
PLCγ
hydrophobic membrane anchor
PIP2
?
Ras
GDP
Ras
GTP
PKB/AKT
antiapoptosis
DAG
+
IP3
Ca 2+ release
PKC
?
Raf1
MEK
ERK
ERK
AP-1, c-Ets
Nucleus

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CHAPTER 2.

Effects of Middle T and Small T Protein Expression on the Induction of DNA Replication Using Plasmids Containing Polyomavirus Origin and Enhancer Sequences

ABSTRACT

Reporter plasmids containing polyomavirus origin and enhancer sequences were constructed and used to study the effect of middle T and small T protein on the induction of DNA replication. Following transient transfections of NIH3T3 cells with reporter plasmids and protein expression vectors, replication levels of reporter plasmids were measured using the *Dpn*I-resistance assay. Replication levels were compared (both in the presence and absence of middle T or small T protein) among plasmids containing a complete enhancer, partial enhancer or no enhancer. Results from these experiments show a middle T-dependent induction of DNA replication in the range of 2 to 4-fold. To study whether or not the ability of middle T or small T protein to induce plasmid DNA replication would correlate with its ability to induce gene expression, a plasmid was also

constructed from which replication and reporter gene expression could be measured simultaneously. Results from experiments comparing this plasmid to others containing enhancer sequences demonstrate that the effect of middle T on reporter gene expression depends strongly on the specific enhancer sequences used. Further results indicate that when using one plasmid to measure both replication and gene expression, middle T induces replication and gene expression to similar levels of approximately 3 to 5-fold in the presence of a complete enhancer. Small T has little, if any, effect on either replication or gene expression under the same conditions. Finally, NIH3T3 cells were used to create a stable cell line containing integrated polyomavirus origin and enhancer sequences. This cell line was used to test the possible influence of cellular chromatin structure on the ability of middle T or small T protein to induce DNA replication. The data obtained from these experiments confirm that middle T protein induces modest levels of polyomavirus DNA replication and suggest that chromatin structure may influence the level of induction.

INTRODUCTION

Polyomavirus is a small, double-stranded DNA tumor virus that has been used extensively to study mechanisms of eukaryotic DNA replication and transcription. The viral genome encodes three structural proteins (VP1, VP2 and VP3) and three regulatory proteins (large T, middle T and small T). The genome also contains a non-coding regulatory region that includes the viral origin of replication, an enhancer and the early and late transcriptional promoters (1,2). Large T is the replication initiator protein. It binds specific sequences within the origin where it melts and unwinds the DNA strands (3-5).

The viral enhancer regulates both the early and late transcriptional promoters. It is also required for viral DNA replication (6). The enhancer contains binding sites for several cellular transcription factors including AP1 and c-ets (7,8). Upon binding the enhancer, these proteins are able to mediate transcriptional activation (9).

Middle T (mT) is the transforming protein of polyomavirus. Its role in transcriptional activation has been well characterized. At the plasma membrane, middle T forms a stable complex with members of the c-src family of

protein kinases leading to its phosphorylation at key tyrosine residues. The phosphorylated tyrosines provide docking sites for molecules involved in cellular signaling cascades resulting in the activation of transcription factors including AP1 and c-ets (10-15).

The individual roles of middle T and small T (sT) protein in viral DNA replication have not been well defined, although viral mT/sT mutants were shown to have a defect in genome replication (16). Therefore, in this report we have utilized both a transient transfection system and a stable cell line system to study the effects of the expression of middle T and small T protein on the induction of DNA replication using plasmids containing the polyomavirus origin and enhancer sequences. The results demonstrate that middle T protein induces increased levels of polyomavirus DNA replication. Further, this induction is shown to require the enhancer, which suggests a direct role for nuclear transactivators in mediating this effect.

MATERIALS AND METHODS

Plasmid DNA. Plasmid pPyOri-alpha was created by the digestion of pG1xB (17) with *Xho*I followed by cloning of a 23-bp *Xho*I linker containing one unique site for both *Kpn*I and *Not*I. The resulting plasmid pOri(Kpn-Not) was digested with both *Kpn*I and *Not*I. A 47-bp *Kpn*I-*Not*I fragment containing binding sequences for both AP1 and c-ets was synthesized as two oligomers (Macromolecular Structure Facility, Department of Biochemistry, Michigan State University). They were subsequently annealed and then ligated to pOri(Kpn-Not). The resulting plasmid, pPyOri-alpha, was sequenced (Sequencing Facility, Department of Botany and Plant Pathology, Michigan State University) to confirm sequence integrity and orientation.

Plasmid pG0 (a gift from P. Chambon) contains the polyomavirus origin and has been described elsewhere (17). Plasmid pPyOri-Enh was created by digesting pG0 with *Xho*I followed by ligating a PCR-amplified 227-bp *Xho*I fragment containing the complete Py enhancer into the same site in pG0.

Plasmid pUHC13-3 contains the firefly luciferase gene whose transcription is driven by the hCMV minimal promoter (a gift from M. Gossen and H. Bujard) and has been described elsewhere (18). Plasmid pPyOri-Enh/Luc was created by digesting pUHD13-3 with *Xho*I followed by ligating a PCR-amplified 327-bp *Xho*I fragment containing the complete Py origin of replication and enhancer into the same site in pUHD13-3.

Plasmid p2xAP1-Luc (a gift from L. McCabe) contains two tandem copies of the human collagenase AP1 binding site fused immediately upstream of the firefly luciferase gene whose transcription is driven by the rat prolactin promoter.

Plasmid pMEX expression vector (a gift from R. Schwartz) drives expression of cloned sequences using the murine 5′ MSV-LTR. pMEX-mT was created by digesting pMEX within its multiple cloning site with *Bam*HI followed by ligating a PCR-amplified 1301-bp *Bam*HI fragment containing the complete Py mT cDNA into the same site in pMEX.

The expression plasmids pCMV-LT and pCMV-mT contain cDNA corresponding to either LT or mT and were gifts from B. Schaffhausen.

Cell lines. NIH3T3 mouse fibroblast cells were used for these experiments. Cell lines were maintained in a humidified incubator at 37°C with 5% CO_2 according to standard culture techniques. The growth medium for cell culture was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated calf serum and penicillin-streptomycin.

NIH3T3-OEL (origin/enhancer/luciferase) was created by co-transfection of pPyOri-Enh/Luc expression plasmid with a selection plasmid for hygromycin resistance, a mammalian selectable marker. Forty-eight hours post-transfection, cells were re-plated at low density and hygromycin B (Sigma) at a concentration of was added for selection. Resistant clones were selected, expanded and screened for luciferase activity to confirm the integration of pPyOri-Enh/Luc sequences.

Transfection procedure. Cells grown exponentially were seeded at $2x10^5$cells on 35-mm-diameter tissue culture dishes. After seeding, cells were incubated for an additional 18 hours prior to transfection. Transfections were carried out at 37°C, 5% CO_2 for 6-8 hours using the lipofectamine reagent system (Gibco-BRL) following the manufacturers' recommendations. Afterwards, the

transfection mixture was carefully aspirated from each tissue culture dish and the cells were refed with medium containing 0.5% serum. Low molecular weight DNA was isolated by the Hirt method at 48 hours from the start of the transfection. After phenol/chloroform extraction and 95% ethanol precipitation, each DNA pellet was resuspended in 30ul of TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA, pH 8.0) and stored at -20° C.

Replication assay using non-integrated reporter plasmids. Selection screening for the replicated plasmid molecules was achieved through the use of the methylation-sensitive restriction endonuclease *Dpn*I (19). This enzyme recognizes and cleaves DNA within the sequence "GATC" when the "A" is methylated. DNA cloned into shuttle vectors remains methylated at the "A" of "GATC" sequences by the enzyme DAM methylase while those vectors are propagated in DAM^+ E.coli. When the plasmids are transfected into eukaryotic cells and begin to replicate, the DNA looses its bacterial methylation pattern due to the lack of a DAM methylase enzyme in eukaryotes. This plasmid DNA then becomes resistant to *Dpn*I cleavage and can, therefore, be separated from non-replicated molecules based on the difference in sensitivity to *Dpn*I. Where indicated, an

additional restriction enzyme, *Hind*III, was used to linearize replicated plasmid DNA. One-third of the DNA sample obtained from a single 35-mm-diameter dish was electrophoresed through a 1.0% agarose gel. Electrophoresis was carried out at 0.8volt/cm for 10-12 hours in Tris-borate/EDTA buffer, pH 8.0. The DNA was denatured in-gel with 0.5M NaOH/1.5M NaCl and transferred to a nylon membrane (Hybond-N, Amersham). The DNA was immobilized on the membrane by UV-crosslinking (12,000 microjoules, 254nm) and probed with ^{32}P-labeled DNA from the plasmid pG0 at 65°C in a hybridization buffer containing 10% dextran sulfate/5X SSPE/5X Denhardt's solution/0.5% (w/v) SDS. The radioactive probe was synthesized using a multiprime DNA labeling kit (Amersham) with [^{32}P]-dCTP. Probe specific activity was routinely 1-3 x 10^9 cpm/ug. Replication bands were quantitated using the STORM phosphorimaging system (Molecular Dynamics). Fold increases were calculated as the ratio of (+)middle T to (-)middle T phosphorimaging counts from Table 1.

Replication assay using an integrated reporter plasmid. NIH3T3-OEL cells were co-transfected with the appropriate expression plasmid(s) for 6-8 hours using Lipofectamine reagent (Gibco-BRL). Forty-eight hours post-

transfection the cells were lysed and total cellular DNA was isolated using Buffer A (10mM TrisCl, pH 7.6, 10mM EDTA, 0.2%SDS, 50ug/ml proteinase K). After phenol/chloroform extraction and 95% ethanol precipitation, each DNA pellet was resuspended in 200ul of TE buffer (10 mM Tris, pH 8.0 and 1 mM EDTA, pH 8.0) and stored at -20° C. Replication of the integrated reporter plasmid was measured by incubating the DNA samples with a restriction endonuclease that either does not cut (*Nhe*I) or cuts only once (*Eae*I or *Hind*III) within the plasmid. *Dpn*I was included to digest the non-replicated expression plasmid DNA in the samples. Electrophoresis, Southern blotting and hybridization followed the same procedures used for the analysis of non-integrated reporter plasmid replication except for the following modifications: (1) electrophoresis was carried out at lower voltage and for >24 hours to improve band separation (2) the DNA was depurinated in-gel with 0.25M HCL before the denaturation step in order to increase the transfer efficiency of the high molecular weight DNA (3) The DNA was probed with ^{32}P-labeled DNA from a PCR-amplified fragment containing the complete polyomavirus origin-enhancer region. Replication bands were quantitated using the STORM phosphorimaging system (Molecular Dynamics).

Western blot analysis. Protein extracts were isolated either from intact cells or from Hirt pellets by resuspension in protein sample buffer (Bio-Rad) and boiling for 5 minutes. Aliquots were electrophoresed through 10% SDS-PAGE gels and then electrotransferred to (+)charged nylon membranes (Amersham). A polyclonal antiserum specific to an epitope shared by LT, mT and sT proteins was used in conjunction with the Super Signal West Pico enhanced chemiluminescent system,ECL (Pierce), to detect polyoma early proteins.

Luciferase Assay. Protein extracts were prepared and the assay performed using the reporter lysis buffer and luciferase assay kit (Promega) and a Turner TD-20e luminometer according to the manufacturers' recommendations. Fold increases were calculated as the ratio of (+)middle T to (-)middle T luciferase light units.

RESULTS

Effect of middle T protein expression on the replication of pPyOri-alpha. To test the effect of mT protein on DNA replication, a reporter plasmid, pPyOri-alpha, containing sequences from the polyomavirus alpha-core enhancer domain was used in a transient transfection. Replication levels were determined by using the *Dpn*I-resistance assay. NIH3T3 cells were transfected with pPyOri-alpha and pCMV-LT, a LT protein expression vector. Forty-eight hours post-transfection low molecular weight DNA and protein extracts were collected. The DNA samples were treated with *Dpn* I (to digest non-replicated plasmid DNA) and *Hind* III (to linearize replicated plasmid DNA). Replicated DNA bands were separated by electrophoresis and analyzed by Southern blot as described in materials and methods. Protein extracts of cells transfected in parallel were analyzed by western blot using anti-T antigen antiserum as described in materials and methods. Replication using this reporter plasmid was dependent on the presence of LT protein (data not shown). The level of replicated DNA increased in a dose dependent manner in response to LT protein levels (figure 2.1, panel A).

Protein analysis (figure 2.1, panel B) demonstrated the expression of LT protein. In addition, transfection experiments using the reporter plasmid pG0, which contains the polyoma origin but not the enhancer, verified that under the conditions tested, replication was dependent upon the presence of enhancer sequences (figure 2.3, "no enhancer" column).

Co-transfection of NIH3T3 cells with pCMV-LT and pCMV-mT increased the replication of the reporter plasmid pPyOri-alpha by 1.3-fold (figure 2.2, panel A). Protein analysis demonstrated the expression of both mT and LT proteins (figure 2.2, panel B). Similar results were obtained in several repeat experiments (Table 1), suggesting that the increase in DNA replication observed was indeed a result of mT protein expression and not simply an artifact related to variations in LT protein levels.

Over the course of several replication experiments, it was observed that co-transfection of mT and LT expression plasmids would, on occasion, result in increased or reduced levels of expression of both proteins compared to levels of detectable protein measured when either plasmid was transfected alone. To minimize any effects of variations in protein levels, subsequent replication assays were carried

out using pMEX-mT. This protein expression vector has two advantages over the pCMV-mT vector: (1) it contains a different promoter from that of the pCMV-LT expression vector and (2) it produces higher levels of mT protein expression using significantly lower concentrations of plasmid compared to pCMV-mT.

The complete polyomavirus enhancer contains binding sites for mT-responsive transcription factors outside of the alpha-core domain. Therefore, replication was measured using reporter plasmids containing either the full enhancer (pPyOri-Enh) or the alpha-core domain (pPyOri-alpha). The level of replication following co-transfections using pMEX-mT showed an increase of 1.2 to 1.4-fold using either reporter plasmid (figure 2.3, upper panel) compared to co-transfections without pMEX-mT. Table 1 presents replication data from several independent experiments using pPyOri-alpha as the reporter plasmid. Protein analysis demonstrated the expression of both LT and mT protein (figure 2.3, lower panel). When replication was measured following co-transfections using the reporter plasmid pG0, a faint replicated band was observed in the absence of mT protein (figure 2.3, upper panel, "No enhancer" column). This band did not increase in intensity in the presence of

mT, indicating that mT's effect on replication requires the viral enhancer.

Taken together, these results demonstrate a modest but reproducible induction of DNA replication by middle T protein.

Effect of expression of middle T protein on gene expression using p2xAP1-Luc reporter plasmid. The modest effect of mT protein on replication appeared to contrast with previous results demonstrating higher levels of increase in gene expression using reporter plasmids containing only an AP1 binding site as the enhancer element. Therefore, we wanted to determine if mT protein expressed in this system could activate gene expression using a similar reporter plasmid containing two tandem copies of the AP1 binding site as the enhancer element. NIH3T3 cells were co-transfected with p2xAP1-Luc and pMEX-mT. Forty-eight hours post-transfection, extracts were collected for both protein analysis and to assay for luciferase activity. The presence of mT protein correlated with an increase in p2xAP1-Luc gene expression of 17-fold (figure 2.4a, lane 2), demonstrating that middle T protein can activate gene expression using a reporter containing two tandem copies of the AP1 binding site. This increase in gene expression in response to mT protein was larger than

the increase in plasmid DNA replication in response to mT protein (figs 2.2 and 2.3).

Middle T protein activates both DNA replication and gene expression using a dual reporter plasmid system. In the previous experiments, gene expression and DNA replication were assayed using separate plasmid vectors. We next tested whether mT protein would have a more pronounced effect on DNA replication if measured along with gene expression on the same plasmid vector. A dual reporter plasmid, pPyOri-Enh/Luc, was constructed and NIH3T3 cells were co-transfected with either pPyOri-Enh or pPyOri-Enh/Luc and pMEX-mT. Forty-eight hours post-transfection low molecular weight DNA and protein extracts were collected and analyzed. The level of increase in plasmid replication in the presence of mT protein was similar (1.4-fold) using either pPyOri-Enh or the dual reporter pPyOri-Enh/Luc (figure 2.4b, panel A, compare lanes 3-4 and 5-6). Protein analysis (figure 2.4b, panel B) demonstrated the expression of LT and mT. Middle T-dependent gene expression measured using pPyOri-Enh/Luc increased 4-fold (figure 2.4a, lane 4) compared to gene expression in the absence of middle T. Interestingly, the increase in mT-dependent gene expression was reduced by 4-fold using the dual reporter plasmid versus a plasmid reporter measuring gene expression

alone (figure 2.4a, compare 2xAP1-Luc and pPyOri-Enh/Luc). As an additional test, the complete polyomavirus enhancer/origin sequences were added to p2xAP1-Luc, creating the dual reporter plasmid pPyOri-Enh/2xAP1-Luc. Plasmid replication and gene expression was then measured in the presence or absence of mT protein using this vector. The results shown in figure 2.4b (panel A, lanes 1-2) indicate that plasmid replication increased 1.7-fold in the presence of middle T and appeared to be unaffected by the contextual placement of the enhancer-origin sequences. Juxtaposing the otherwise highly mT-responsive expression cassette [2xAP1-Luc] immediately downstream of the complete polyomavirus enhancer/origin resulted in a middle T-dependent increase in gene expression of 5-fold (figure 2.4a, lane 6) compared to gene expression measured using the same reporter but in the absence of middle T. This represented a 4-fold reduction in gene expression comparing p2xAP1-Luc and pPyOri-Enh/2xAP1-Luc, which was similar to that seen with pPyOri-Enh/Luc. The results suggest that under these experimental conditions "promoter context" can influence the level of enhancer-mediated, mT protein-dependent gene expression but has little effect on the level of plasmid DNA replication.

Polyoma enhancer/origin sequences confer orientation-independent activation of DNA replication and gene expression. In the wild-type A2 (WTA2) strain of polyomavirus the enhancer/origin sequences are arranged such that the enhancer element is distal to the early viral promoter (see figure 1.1). To test the effect of enhancer orientation on replication and gene expression two versions of the dual reporter plasmid pPyOri-Enh/Luc were constructed. In the (+)orientation, the enhancer/origin sequences were arranged such that the enhancer element is distal to the promoter driving expression of the luciferase reporter gene (this arrangement parallels that seen in WTA2). In the (-)orientation, the enhancer/origin sequences were inverted. Sub-confluent NIH3T3 cells were co-transfected with either (+) or (-) versions of pPyOri-Enh/Luc in the presence or absence of mT protein. DNA and protein extracts were collected and analyzed.

Results shown in figure 2.5a (upper panel) indicate that the increase in plasmid replication in response to mT protein expression using (+)pPyOri-Enh/Luc was 3.0-fold versus 2.7-fold using (-)pPyOri-Enh/Luc. Protein analysis demonstrated no difference in the level of expression of LT

and mT protein (figure 2.5a, lower panel). Thus, the results indicate that mT-dependent plasmid DNA replication is independent of the orientation of the polyomavirus enhancer/origin sequences. As shown in figure 2.5b, the increase in mT-dependent gene expression was 5-fold using (+)pPyOri-Enh/Luc and 4-fold using (-)pPyOri-Enh/Luc. Thus, mT-dependent gene expression is also independent of the orientation of the polyomavirus enhancer/origin sequences.

Small T protein expression does not efficiently induce DNA replication or gene expression measured using dual reporter plasmids. Small T protein was tested for its ability to induce either DNA replication or gene expression. Dual reporter plasmids were co-transfected with pCMV-LT and with or without pCMV-sT. Expression of small T in this experiment induced plasmid replication 1.5-fold and gene expression 2.0-fold (figures 2.6a and 2.6b). However, the results of repeat experiments showed no induction by small T (data not shown). Since the results with small T were not conclusive, a small T effect on DNA replication cannot be ruled out at this time.

Effect of middle T protein expression on the replication of an integrated reporter plasmid. To examine the effect of middle T protein on a DNA template with a cellular pattern of chromatin organization a cell line, NIH3T3/OEL, was constructed which contains the stably integrated plasmid reporter pPyOri-Enh/Luc. Since the cellular DNA (and the integrated sequences following one round of replication) is resistant to *Dpn*I, replication induction was identified by comparing the intensity of restriction fragments following expression of different combinations of LT, mT and sT protein. Southern blot analysis of total cellular DNA from NIH3T3/OEL using an origin/enhancer-specific radiolabeled probe suggests that there are two major sites of integration (figure 2.7, lane 5). In this experiment *Nhe*I, a restriction endonuclease that does not cut within the integrated plasmid DNA sequences, was used to digest the cellular DNA samples. In this case, the simultaneous expression of middle T and large T proteins resulted in a significant increase in both the number and intensity of bands representing replicated DNA (figure 2.7, compare lanes 6-8 to lanes 2-4). When restriction endonucleases (*Eae*I and *Hind*III) that cut once within the integrated plasmid DNA sequences were used to digest the cellular DNA samples, the banding pattern became

less complex (figure 2.8, compare lanes 5-6 to lanes 3-4 and lanes 11-12 to lanes 9-10). No significant increase in DNA replication was observed when small T protein was expressed (data not shown) suggesting that only mT protein was able to increase the replication observed using the NIH3T3-OEL cell line. These results provide evidence that middle T protein can increase DNA replication of chromatin templates, presumably through its ability to activate nuclear transcriptional activators.

DISCUSSION

Experiments in this study were designed to test the effect of middle T protein on the induction of polyomavirus DNA replication. The experimental strategy utilized a transient replication assay with test plasmids containing the polyomavirus origin and either the complete enhancer or portions thereof. Results from these experiments revealed an ability of middle T protein to activate DNA replication. The increases observed were modest but reproducible and ranged from 1.5 to 4.0-fold (Table 1.). Activation of DNA replication required the presence of the polyomavirus enhancer as demonstrated in co-transfection experiments comparing the replication of reporter plasmids containing the complete enhancer, a partial enhancer or no enhancer. The absence of the enhancer resulted in very low levels of plasmid replication confirming previous reports that efficient polyomavirus DNA replication requires the enhancer (20-23). This low level of replication could not be induced to higher levels upon expression of middle T protein. Thus, the effect on DNA replication observed in this study is not a result of an ability of middle T to cause functional changes in large T protein. Induction of replication was also studied in an NIH3T3 cell line

containing a stably integrated reporter plasmid by measuring replication following transfection and expression of large T and either middle T or small T proteins. Results from these experiments show that middle T protein can activate DNA replication from chromatin templates and that the degree of activation appears to be more pronounced than that seen when using plasmid templates in transient replication assays.

It is generally appreciated that transcription is repressed by chromatin structure and that activation domains of transcription factors can relieve this repression by inducing chromatin remodeling (24-26). Several lines of evidence show that a similar mechanism may allow transactivators to enhance DNA replication. One report found that in vitro replication of SV40 genomes was inhibited by allowing pre-assembly of the DNA into chromatin. This inhibition could be overcome by the addition of the transcriptional activator NF1 and the inclusion of NF1 binding sites in the template DNA (27). In subsequent experiments using GAL4-VP16 fusion proteins, it became clear that this ability to induce replication of repressed chromatin templates was not limited to NF1 and appeared to require only an acidic transcriptional activation domain (28). Similar induction of replication

from repressed chromatin was observed using bovine papillomavirus DNA and either the E2 viral transactivator or a GAL4-VP16 fusion protein (29). Several reports have established a physical connection between a number of transactivators and the co-adaptor p300/CBP. p300/CBP has intrinsic histone acetyltransferase (HAT) activity. Of particular relevance to the current study are reports that demonstrate an interaction between AP1/c-jun and p300/CBP (30-32). One important consequence of such an interaction is that the HAT activity of p300/CBP is brought to the chromatin template where it can modify nucleosomal histones and facilitate chromatin remodeling. This provides another potential mechanism for how transactivators can induce DNA replication. These effects of transactivators on DNA replication are not restricted to viral origins since it has recently been shown that transactivator-mediated chromatin remodeling can also activate cellular origins of replication (33). Thus, the requirement of the polyomavirus enhancer to activate DNA replication could reflect a chromatin remodeling mechanism of action mediated by middle T-activated transcriptional activators that bind within the enhancer.

In the "Review of Literature" section some limitations in testing middle T protein effects on DNA replication

using viruses were discussed. Recently, our laboratory has designed a novel experimental approach that allows the use of viruses to study middle T protein effects on DNA replication. In these experiments, NIH3T3 cells were first transfected with a middle T protein expression vector. After allowing time for cell recovery and expression of middle T protein, the cells were infected with the mT/sT defective virus A185. Preliminary results using this experimental strategy show that viral DNA replication is increased in the presence of middle T protein in the range of 4 to 10-fold. This suggests that the effect of middle T protein on DNA replication is more pronounced when measured using viral genomes versus plasmid DNA, possibly due to different patterns of nucleosomal organization (chromatin structure) between the two DNA templates.

In a study using a replication-competent test genome, pPyLT, lacking the information for functional middle and small T proteins (34) it was reported that following co-transfection of viral genomes separately expressing either middle or small T protein, small T protein was able to increase replication approximately 3-fold while mT protein increased replication 1.5-fold (35). Their results with small T protein differ from those obtained in the present study. However, the design of their experiments did not

include protein analysis. Since protein expression was not measured, the amount of LT, mT and sT-protein present and whether or not those levels fluctuated when co-transfections were performed is not known. Fluctuations in protein expression levels during the early stages of the present study (particularly LT-protein) led to replication artifacts that could be misinterpreted as being mediated by middle T or small T protein. One hypothesis for this variability was that promoter competition for limiting cellular factors (made more dramatic when differing concentrations of each plasmid were used in a co-transfection) resulted in one plasmid having an advantage for expression by virtue of the complement of factor binding sites present in its promoter/enhancer. It is possible, therefore, that the small T protein effect on replication observed by Berger and Wintersberger resulted from fluctuations in LT protein expression. The reporter and expression plasmids used in the Berger and Wintersberger study may also have contributed to the difference observed with small T protein. The pPyLT reporter they used to measure DNA replication contains a complete copy of the polyomavirus genome with a deletion of the large T intron. The middle T and small T expression plasmids also contain complete copies of the polyomavirus

genome with deletions in the appropriate intron to allow for expression of only one early protein. Regulation of gene expression for these plasmids uses the viral enhancer and promoter. The replication test plasmids used in the current study contain only the core origin and enhancer sequences. The expression plasmids used in the current study contain cDNA copies of each early gene. Polyomavirus promoter/enhancer sequences are not used to regulate gene expression of these plasmids. Therefore, it is possible that inclusion of substantial portions of polyomavirus sequences in both the replication test plasmids and the expression plasmids in the Berger and Wintersberger study may have had unexpected effects on the replication levels of the test plasmid that were not fully appreciated at the time those experiments were conducted. Another factor to consider is differences in cell lines. Mouse NIH3T3 cells were used in this study, whereas mouse 3T6 cells were used in the Berger and Wintersberger study. In that study, the authors reported (unpublished observation) that using different mouse fibroblast cell lines affected the extent to which small T protein could stimulate the replication of pPyLT, presumably due to a cellular component(s) that may be limiting in the absence of small T protein. Thus, differences in host cellular factors between the two cell

lines could also explain the conflicting results obtained with small T protein in this study versus the previously reported data of Berger and Wintersberger.

Gene expression was measured using separate test plasmids containing a luciferase reporter and either the complete enhancer, portions thereof, or tandem copies of the DNA binding site for the middle T-responsive transactivator, AP1. Dual reporter plasmids were also constructed in order to measure DNA replication and gene expression using the same vector. In this study, middle T protein was shown to be capable of enhancing gene expression in a dose-dependent manner using the reporter vector p2xAP1-Luc, which contains a duplicated AP1 binding site (data not shown). This result is in agreement with findings from previous studies. Middle T protein has been shown to activate transcription of cellular genes whose promoters contain AP1 binding sites (36). Middle T protein is known to induce a cellular signaling cascade of phosphorylation events resulting in the activation of several transcriptional activators including AP1 (as judged by gel mobility shift assays and phosphopeptide mapping) (36,37). In turn, activated AP1 has been shown to increase gene expression using plasmids containing AP1 binding sites linked to a CAT-reporter gene cassette (36). Results from

the current study confirm those obtained in previous reports and provide evidence that mT protein expressed in this system is functional for signaling. Expression of middle T protein led to increases in the level of gene expression ranging from 15 to 25-fold when using a reporter plasmid [p2xAP1-Luc] containing a duplicated AP1 binding site. Comparing reporter gene expression levels using this reporter versus dual reporter plasmids from which DNA replication and gene expression could be measured simultaneously, we observed a 3-fold reduction in gene expression from the dual reporter plasmids. One possible explanation for this observed difference in gene expression is that in transfections of a dual reporter plasmid there may be competition among the replication and transcription machinery for limiting common cellular factor(s) necessary for either event. Since large T protein is required for the initiation of replication complex formation, in its absence replication does not occur. Therefore in the absence of large T protein, mT-induced gene expression should be increased compared to levels of gene expression in the presence of large T protein. To test this hypothesis, a co-transfection experiment was carried out using a dual reporter measuring mT-induced gene expression both with and without large T protein expression. Results indicate that

gene expression from the dual reporter is not increased in the absence of large T protein. Another possibility is that differences in the plasmid backgrounds used to construct the reporters may have affected gene expression levels. This, however, is not likely since one of the dual reporter plasmids was constructed in the background of p2xAP1-Luc but does not share its high levels of mT-mediated gene expression. Alternatively, the polyomavirus enhancer may contain sequences that confer repression of transcription in NIH3T3 cells. Constructing the p2xAP1-Luc reporter with an insert of multiple copies of only the AP1 binding site may have removed repressive sequences thereby producing a vector more sensitive to AP1 activation. A test of this hypothesis would be to construct a new dual reporter plasmid without the polyomavirus enhancer (pPy2xAP1-Ori/Luc), but with mT-responsive, transcription factor binding sites (e.g., AP1) linked to a polyomavirus origin of replication and a gene expression cassette. Transfection experiments using the new dual reporter plasmid should result in activation of gene expression to the higher levels seen when using the reporter plasmid p2xAP1-Luc. There is at least one report in the literature that lends support to the hypothesis that the enhancer contains sequences that may act in a repressive manner under certain

conditions (38). In that report, expression of an activated form of c-jun protein (one of the two members of the AP1 complex) was able to cause transcriptional repression of the polyomavirus enhancer, albeit in a cell-type specific manner. Repression was shown to be independent of both enhancer distance and the two AP1 binding sites within the enhancer. Expression of the same activated c-jun protein led to dose-dependent activation of a reporter containing four copies of the AP1 binding site. These results suggest that a region of the enhancer outside of the AP1 binding sites is responsible for mediating the repressive effect.

In summary, the results from this study demonstrate an ability of middle T protein to induce increased levels of DNA replication using both a transient transfection assay system and an integrated cell line assay system. The induction of replication by middle T required viral enhancer sequences. The induction of replication by middle T was not affected by the orientation of the enhancer sequences with respect to the viral origin.

APPENDIX B

Chapter 2 Figures

Figure 2.1. Large T-dose dependent reporter plasmid replication. (A) Southern blot analysis of replicated DNA using the reporter pPyOri-alpha. Lanes 1-4 cells were co-transfected with 25ng, 75ng, 225ng and 675ng pCMV-LT respectively. Band shown is the full-length HindIII-linearized DpnI-resistant reporter hybridized with a ^{32}P-dCTP labeled pG0 probe. Values below each band represent fold increase in replication with Lane 1 arbitrarily set to 1. (B) Western blot analysis of protein expression levels following co-transfection of NIH3T3 cells using the plasmids indicated in text. Proteins were detected using a polyclonal rat anti-polyoma T antigen primary antiserum and a goat anti-rat horseradish peroxidase labeled secondary antibody. Membranes were then processed using an enhanced chemiluminescent detection system (ECL). The polyomavirus early proteins are indicated by arrows. Lanes 1-4 cells were co-transfected with 25ng, 75ng, 225ng and 675ng pCMV-LT respectively.

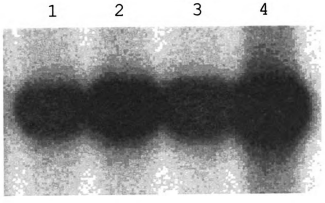
A
1
2
3
4
1
1.5
1.7
2.2
Fold
Replication
Increase
B
(+) control
(-) control
1
2
3
4
large T
middle T

Figure 2.2. Middle T protein activation of DNA replication using a reporter plasmid containing enhancer sequences. (A) Southern blot analysis of replicated DNA using the reporter pPyOri-alpha. In addition to pCMV-LT and the reporter plasmid pOri-alpha, lanes 1 and 2 were co-transfected using pMEX-mT. Southern blot analysis was as described in the legend to figure 2.1. (B) Protein analysis by western blot. Detection of polyoma T antigens was as described in the legend to figure 2.1. Arrows indicate both large T and middle T specific bands.

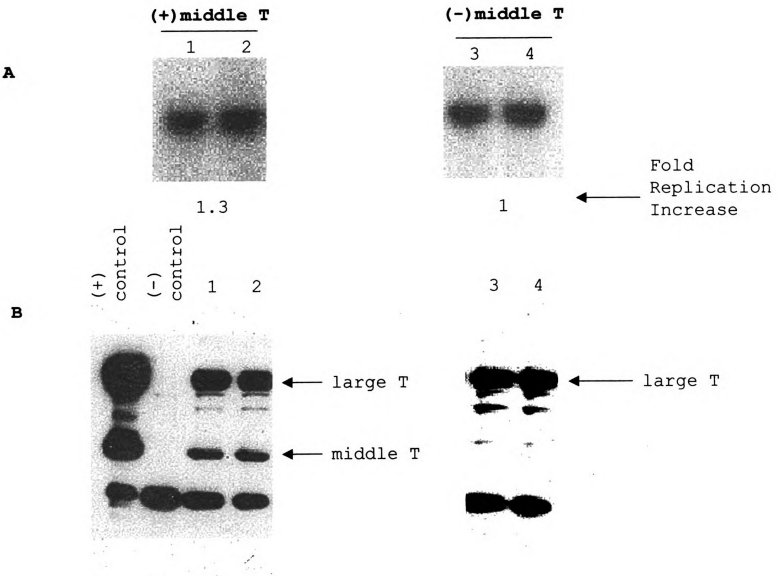
(+)middle T
(-)middle T
A
1
2
3
4
1.3
1
Fold
Replication
Increase
(+) control
(-) control
B
large T
middle T
large T

Figure 2.3. Middle T protein activation of DNA replication using three reporter plasmids containing distinctly different enhancers. In the upper panel, headings indicate which reporter plasmid was used. (+) lanes included pMEX-mT. Fold replication increase induced by mT is indicated below each set of replicated bands. "n/c" denotes no change. In the bottom panel, protein analysis was by western blot as outlined in figure 2.1. "no" heading denotes "no enhancer". Lane (c) is a positive control protein extract from a wild-type polyomavirus infection.

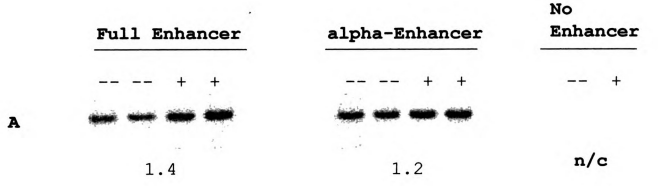
Full Enhancer
alpha-Enhancer
No Enhancer
-- -- + +
-- -- + +
-- +
A
1.4
1.2
n/c
B
(c)
full
alpha
no
large T
middle T

Figure 2.4a. Activation of gene expression by middle T protein using a luciferase reporter plasmid. Co-transfections were carried out using p2xAP1-Luc with or without pMEX-mT. The luciferase assay was carried out as described in materials and methods. Fold increase in gene expression is expressed as the +mT/-mT ratio of the absolute light units obtained from the assay.

Fold Activation
0
2
4
6
8
10
12
14
16
18
2xAP1-Luc
2xAP1-Luc + mT
pPyOri-Enh/Luc (-)mT
pPyOri-Enh/Luc (+)mT
pPyOri-Enh/2xAP1-Luc (-)mT
pPyOri-Enh/2xAP1-Luc (+)mT

Figure 2.4b. Middle T protein activation of DNA replication using a dual reporter plasmid. Upper panel (A), Southern blot analysis of replicated DNA using both single and dual reporter plasmids. In lanes 1-2 and 5-6 replication was measured using the dual reporters pPyOri-Enh/2xAP1-Luc and pPyOri-Enh/Luc respectively. Odd numbered lanes are (-) middle T while even numbered lanes are (+) middle T. Fold replication increase induced by mT is indicated below each set of replicated bands. Lower panel (B), Protein analysis by western blot. Detection of polyoma T antigens was as described in the legend to figure 2.1. Arrows indicate both large T and middle T specific bands.

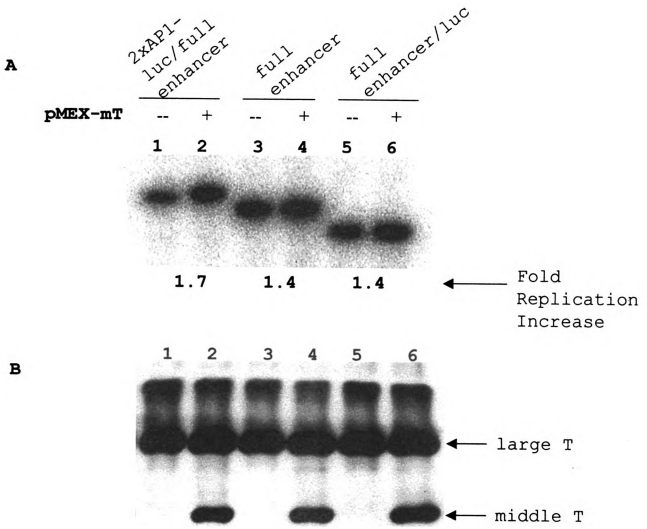
A
2xAP1-luc/full enhancer
full enhancer
full enhancer/luc
pMEX-mT
-- + -- + -- +
1 2 3 4 5 6
1.7 1.4 1.4
Fold Replication Increase
B
1 2 3 4 5 6
large T
middle T

Figure 2.5a. Middle T protein activation of DNA replication using two dual reporter plasmids. Upper panel, Southern blot analysis of replicated DNA using, (A) dual reporter in (+)orientation and (b) dual reporter in (-)orientation. Lanes 1-2 include the expression of middle T protein. Fold replication increase induced by mT is indicated below each set of replicated bands. Lower panel (B), Protein analysis by western blot. Detection of polyoma T antigens was as described in the legend to figure 2.1. Arrows indicate both large T and middle T specific bands.

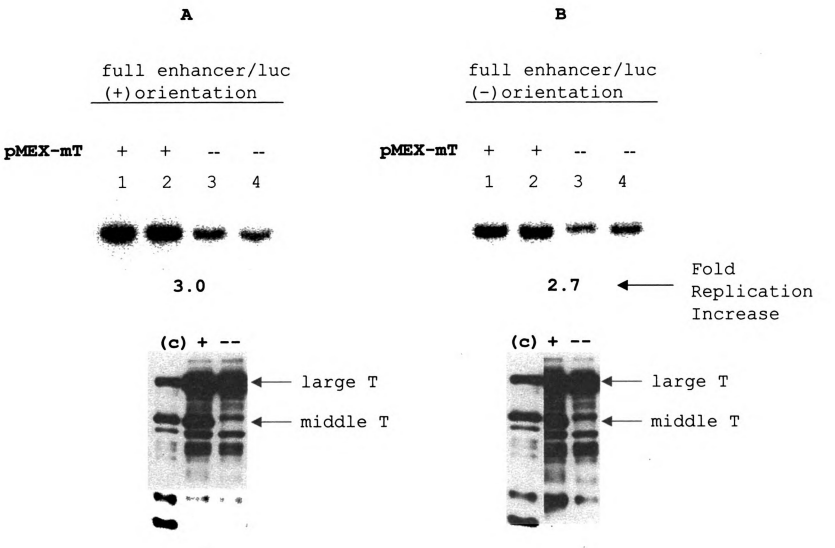
A
full enhancer/luc
(+)orientation
pMEX-mT + + -- --
1 2 3 4
3.0
(c) + --
large T
middle T
B
full enhancer/luc
(-)orientation
pMEX-mT + + -- --
1 2 3 4
2.7
Fold
Replication
Increase
(c) + --
large T
middle T

Figure 2.5b. Activation of gene expression by middle T protein using dual reporter plasmids. Co-transfections were carried out using the indicated reporter with or without pMEX-mT. The luciferase assay was carried out as described in materials and methods. Fold increase in gene expression is expressed as the +mT/-mT ratio of the absolute light units obtained from the assay.

Fold Activation
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Full Enhancer/Luc (+orientation) (-)pMex-mT
Full Enhancer/Luc (+orientation) (+)pMex-mT
Full Enhancer/Luc (-orientation) (-)pMex-mT
Full Enhancer/Luc (-orientation) (+)pMex-mT

Figure 2.6a. Effect of small T protein on the induction of DNA replication using dual reporter plasmids. Upper panel, Southern blot analysis of replicated DNA using the indicated reporter. Lanes 1-2 include the expression of small T protein. Fold replication increase induced by sT is indicated below each set of replicated bands. Lower panel (B), Protein analysis by western blot. Detection of polyoma T antigens was as described in the legend to figure 2.1. Arrows indicate both large T and middle T specific bands.

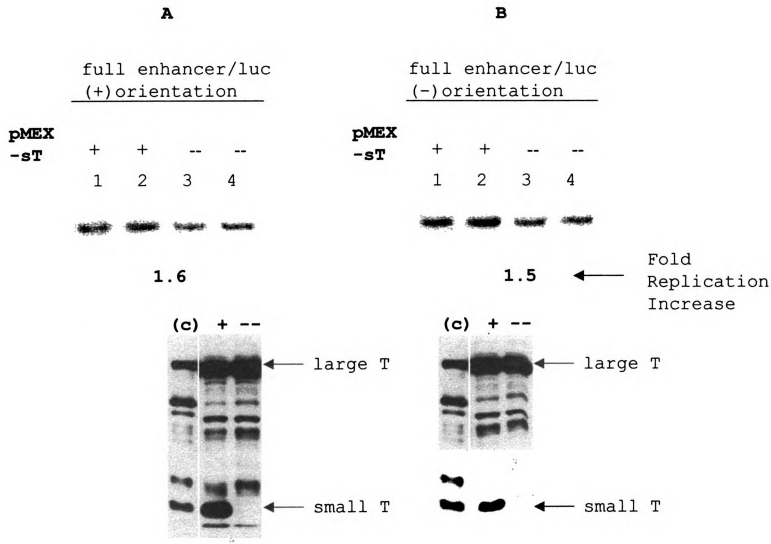
A
full enhancer/luc
(+)orientation
pMEX
-sT
+ + -- --
1 2 3 4
1.6
B
full enhancer/luc
(-)orientation
pMEX
-sT
+ + -- --
1 2 3 4
1.5
Fold
Replication
Increase
(c) + --
large T
small T
(c) + --
large T
small T

Figure 2.6b. Effect of small T protein on the induction of gene expression using dual reporter plasmids. Co-transfections were carried out using the indicated reporter with or without pCMV-sT. The luciferase assay was carried out as described in materials and methods. Fold increase in gene expression is expressed as the +sT/-sT ratio of the absolute light units obtained from the assay.

Fold Activation
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Full Enhancer/Luc (+orientation) (-)cmv-sT
Full Enhancer/Luc (+orientation) (+)cmv-sT
Full Enhancer/Luc (-orientation) (-)cmv-sT
Full Enhancer/Luc (-orientation) (+)cmv-sT

Figure 2.7. Middle T protein activation of DNA replication using the NIH3T3-OEL cell line. Total cellular DNA samples were digested with *Nhe*I + *Dpn*I and then analyzed by Southern blot using a plasmid-specific radiolabeled DNA probe. Large T and middle T protein expression plasmids were transfected in the lanes indicated (+). The asterisks indicate both novel bands and bands that increased in intensity upon expression of middle T protein. Protein analysis by western blot for samples co-transfected with both pCMV-LT and pMEX-mT is shown to the left.

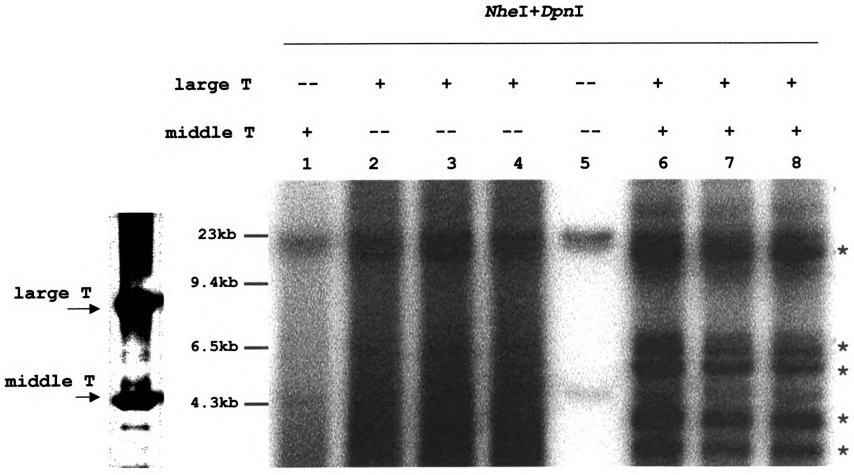
NheI+DpnI
large T -- + + + -- + + +
middle T + -- -- -- -- + + +
1 2 3 4 5 6 7 8
23kb
9.4kb
6.5kb
4.3kb
large T
middle T
*
*
*
*
*

Figure 2.8. Middle T protein activation of DNA replication using the NIH3T3-OEL cell line. Total cellular DNA samples were digested with *Eae*I or *Hind*III + *Dpn*I and then analyzed by Southern blot using a plasmid-specific radiolabeled DNA probe. Large T and middle T protein expression plasmids were transfected in the lanes indicated (+). The asterisks indicate both novel bands and bands that increased in intensity upon expression of middle T protein.

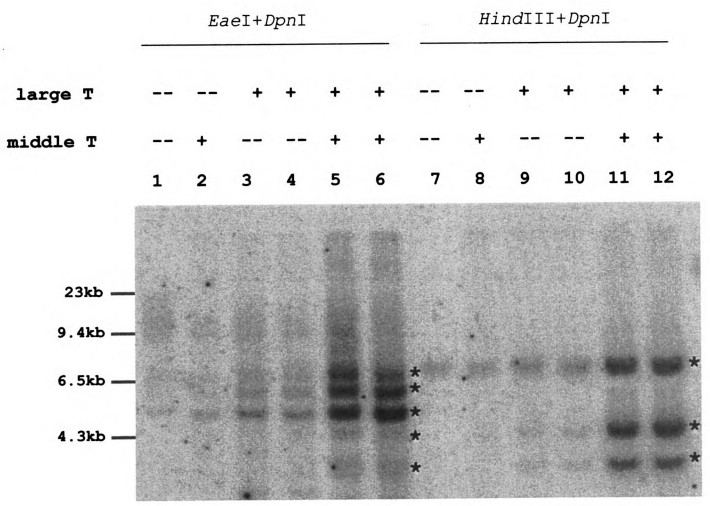
EaeI+DpnI
HindIII+DpnI
large T
middle T
1 2 3 4 5 6 7 8 9 10 11 12
23kb
9.4kb
6.5kb
4.3kb

Table 1. Middle T protein activation of DNA replication. Each sub-table represents repeat experiments with the reporter plasmids indicated in the far left column. Values in (+) and (-) middle T columns were obtained from phosphorimager quantification of replicated DNA bands. Fold increases were calculated as the ratio of mean (+)middle T to mean (-)middle T.

Middle T Activation of DNA Replication (DpnI-resistance assays)

	(-) MIDDLE T	(+) MIDDLE T	FOLD INCREASE
pPyOri-Enh	152618	210770	1.4
pPyOri-Enh	279875, 234450	1009024, 1024433	4.0
pPyOri-Enh	170680, 208174	515654, 470796	2.6
pPyOri-Enh	18858, 23349	49718, 65085	2.7
pPyOri-Enh	70282, 90624	104488, 112203	1.3
pPyOri-Enh	82001, 69820	116742, 79752	1.3
pPyOri-Enh	38291, 33355	50311, 61297	1.6

Mean 2.1 ± 0.94 s.d.

	(-) MIDDLE T	(+) MIDDLE T	FOLD INCREASE
pPyOri-alpha	52861, 49248	58025, 63784	1.2
pPyOri-alpha	59700, 54412	70205, 64116	1.2
pPyOri-alpha	43198, 44543	58600, 58731	1.3
pPyOri-alpha (AMBIS)	22254, 25365	39348	1.7
pPyOri-alpha (AMBIS)	3056, 3484	6738, 7181	2.1

Mean 1.5 ± 0.35 s.d.

	(-) MIDDLE T	(+) MIDDLE T	FOLD INCREASE
pPyOri-Enh/Luc	122330	165862	1.4
pPyOri-Enh/Luc	141925, 94512	354520, 354934	3.0
pPyOri-Enh/Luc (-) orientation	106243, 121583	293327, 312970	2.7

Mean 2.4 ± 0.70 s.d.

	(-) MIDDLE T	(+) MIDDLE T	FOLD INCREASE
2xAP1Ori-Enh/Luc	2196	5485	2.5

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