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THE ROLE OF MIDDLE T ANTIGEN MEDIATED ENHANCER ACTIVITY IN POLYOMA VIRAL DNA REPLICATION AND NEOPLASTIC TRANSFORMATION

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 Public Health

1991

ABSTRACT

THE ROLE OF MIDDLE T ANTIGEN MEDIATED ENHANCER ACTIVITY IN POLYOMA VIRAL DNA REPLICATION AND NEOPLASTIC TRANSFORMATION

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Effects of polyomavirus enhancer on viral DNA synthesis and neoplastic transformation were investigated. Competition assays indicate that NIH-3T3 mouse cells contain limiting factors for polyoma viral DNA replication and that target sequences for these factors are located within the A enhancer element. In the absence of middle T antigen, virus with a duplicated A enhancer element displayed growth advantage over the wild-type A2 enhancer. Enhancer competition was alleviated by middle T antigen, and partially by serum growth factors and/or TPA. This suggests that middle T antigen affect enhancer activity through a signal transduction or a transformation pathway. Given that all natural hr-t isolates have evolved with rearrangements in the same region of the enhancer, we propose it as a

compensatory event for the loss of middle T and/or small T antigens.

Enhancer mediated competition was also observed in nonpermissive FR-3T3 cells, suggesting that rat cells contain limiting enhancer factors. Wild-type virus induced stable transformation was interfered more pronounced than viral DNA replication and abortive transformation. Taken with the observation of low probability for double integration, we propose that integration is a rate limiting step during the process of polyomavirus mediated neoplastic transformation. It can be either due to the existence of limiting amounts of one or several factors required for integration step, or due to the limitation of integration events in the host chromosome. Since both viruses produce normal large T antigen, which is required for integration, it is likely that integration of one viral genome prevents a secondary event.

To my parents

ACKNOWLEDGEMENTS

I would express my deep appreciation and gratitude to Dr. Michele Fluck for her encouragement, invaluable discussions, and financial support throughout my graduate program.

I thank the members of my guidance committee for their advice and interest: Drs. Susan Conrad, Larry Snyder, and John Wang.

I would like to acknowledge Drs. Karen Friderici and Andrea Amalfitano who initiated studying enhancer sequences of hr-t mutants, Makda Fisseha for assistance with data presented in Chapters 2 and 3. I also thank the people in Dr. Fluck's laboratory for their help and shared ideas.

Finally, my special thanks go to my family for their love and constant support, my fiancè' Dr. Perng Kuang Chang for his friendship with great understanding and patience.

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Literature Review

Introduction

The mouse polyomavirus is a member of the papovavirus family (1). Its genome is a double-stranded, covalently-closed circular DNA molecule consisting of 5297 base pairs (2). Viral DNA exists in virion as a minichromosome complexed with cellular histones surrounded by capsid proteins. The structure and histone composition of the viral nucleosomes are indistinguishable from those of the host (3). The polyoma virion is approximately 45 nM in diameter and is composed of three virus-coded proteins, VP1, VP2, and VP3, forming a icosahedral structure. VP1 is the major component of the viral capsid, making up about 75% of the total virion protein (1).

Polyomavirus was first discovered in 1953, and was later shown to cause a wide variety of solid tumors in mice and other rodents (4,5). Although the virus is prevalent, the incidence of tumors in the natural population is quite low. This has been attributed to the immune protection of the host acquired during gestation, low doses of infecting virus, and to the existence of an anti-tumor response. The mechanism for such immunological resistance to tumor formation provides an interesting field to be explored.

In tissue culture systems, a productive infection, also defined as lytic infection, takes place in mouse fibroblast cells. The vast majority of these cells, designated as permissive cells, support viral DNA replication, express all viral gene products, and eventually are lysed and release infectious progeny virus particles. To find a tumor induction process in tissue culture equivalent to that in mouse, other hosts were tested. Hamster and rat cells are semi- and non-permissive, respectively, meaning that viral infection produces much less or no viral particles. Such permissivity seems to be determined by one or more trans-dominant factors of the host, since hybrids from permissive and nonpermissive cells are productively infected (6). Infection of nonpermissive cells results in cellular immortalization and/or neoplastic transformation.

On infection of permissive cells, the virion adsorbs to the cell membrane, penetrates into the nucleus, and then is uncoated (7). The interaction between the virion and its membrane receptor induces the expression of two cellular oncogenes, c-*myc* and c-*fos*, which are important for both viral DNA synthesis and gene expression (8). Three early genes coding for proteins referred to as T antigens are expressed. These proteins are involved in stimulating expression of cellular genes and synthesis of cellular DNA, and in facilitating viral growth. The large T antigen is

required for the initiation of viral DNA synthesis, and regulates the expression of viral early and late genes. Following the amplification of the viral genome, three late genes encoding the capsid proteins are expressed. Virions are then assembled in the nucleus and released after cell lysis (9). An *in vitro* system that replicates polyoma viral DNA in cell-free condition has been established. It requires extracts of mouse cells, circular DNA containing the viral replication origin, and purified polyoma large T antigen (10).

Growth of polyomavirus in nonpermissive cells has provided a useful model system for studying virally induced neoplastic transformation. Host cells respond to viral infection by undergoing DNA synthesis and cell division. A number of properties associated with transformed cells are transiently expressed. However, most of the infected cells soon lose the viral DNA, and revert to their uninfected state. This process during which the viral early genes are expressed is termed "abortive transformation" (11). Eventually, a small portion (<1%) of the infected cells become stably transformed. Integration of viral DNA into the host chromosome and continuous expression of middle T antigen are required for "stable transformation". Transformed cells display reduced requirement for serum, altered morphology with a disrupted cytoskeletal array, loss of contact inhibition, ability to grow in semisolid medium

without surface attachment, and ability to form tumors in syngeneic animals (1,12).

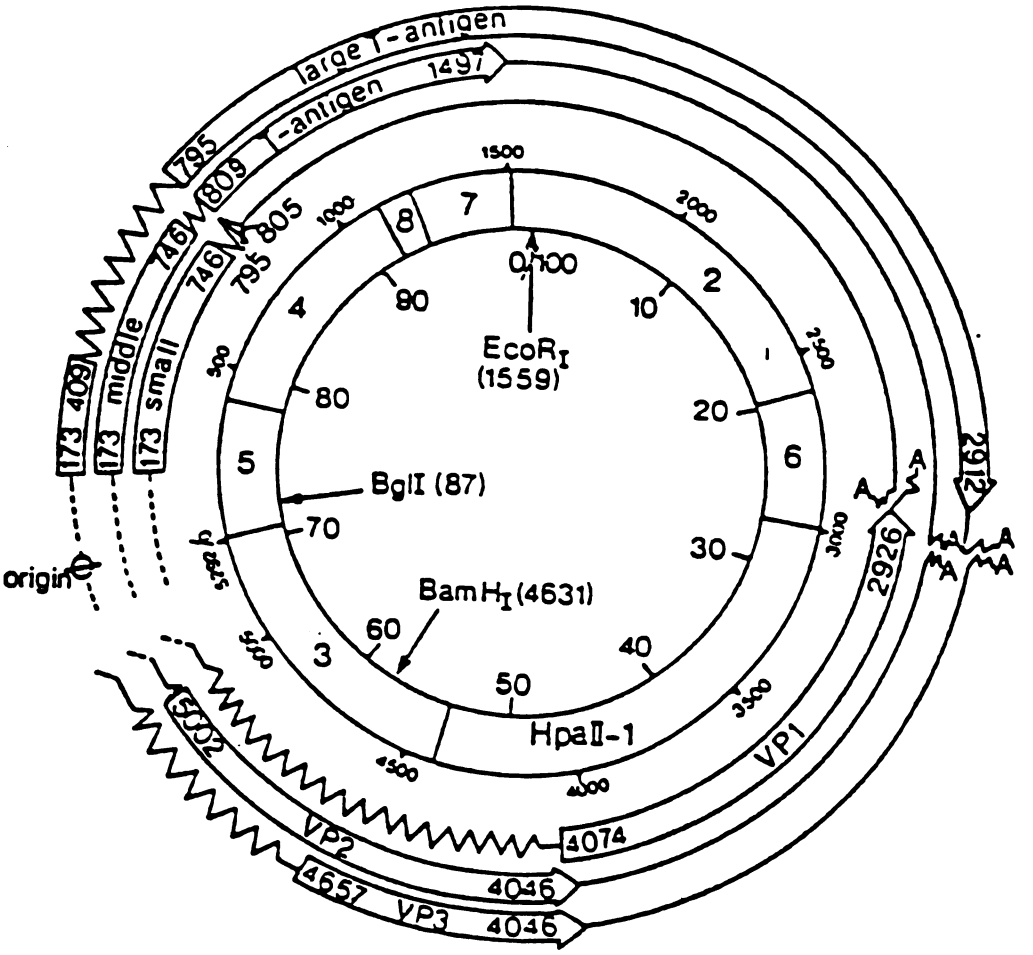
This thesis will address certain specific issues on middle T antigen mediated polyoma enhancer activities, and on stimulation of viral DNA replication and neoplastic transformation by these activities. Therefore, I will emphasize this literature review on the following topics: structural organization of the viral genome, functional analysis of the noncoding regulatory region, functions of the early viral proteins on viral DNA replication, and the mechanisms of cell transformation by polyomavirus.

Structural Organization of the Polyomavirus Genome

The polyomavirus genome, though having a small size, is organized in a way to obtain maximum utility by overlapping genes which are expressed through differential processing of RNA transcripts (Fig.1) (13,14). The viral genome is divided into an early region and a late region, separated by approximately 350 base pairs of a noncoding regulatory region (15). The primary transcripts of early and late RNA are synthesized in opposite direction using each own initiation signals. The early promoter possesses several canonical TATA and CAAT boxes, and the major start sites of early transcripts are at nucleotides 147 and 152 as determined by primer extension and S1 mapping (Fig.2) (16,17), whereas, the late promoter lacks a TATA box, CAAT

Figure 1. Physical map of polyomavirus genome.

The inner circle represents the *Hpa*II/*Msp*I restriction map of polyoma genome with the numbering system of Soeda et al. (15) beginning at the junction of fragments 3 and 5. The noncoding region flanks the origin of DNA replication. The early and late precursor RNAs are spliced as indicated (jagged lines represent introns) to generate the mRNAs for the T antigens or the capsid proteins, respectively. The boxed regions represent the protein coding sequences. The nucleotides of the splicing junctions and of the termini of the coding regions are also shown. The figure is taken from Soeda et al. (15).


large T-antigen
1497
-antigen
795
809
746
805
795
746
746
173 409
173 middle
173 small
origin
EcoR$_I$
(1559)
BglI (87)
BamH$_I$(4631)
HpaII-1
VP1
VP2
VP3
4074
4046
4657
4046
2926
2912
1
2
3
4
5
6
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100

box, or G+C-rich region, which are sequences often associated with the regulation of transcription initiation. Several preferred sites for late transcription initiation span more than 100 nucleotides which results in late RNA with extremely heterogeneous sizes (Fig.2) (18). Termination signals for transcription are located at the 3' end near poly A residues. However, it has been suggested that termination signals are inefficient since small amount of "run through" transcripts larger than late RNA are observed (19). Alternative splicing of the primary early transcript generates three transcripts coding for the early proteins: small, middle and large T antigens (16,20). The late primary transcript is also differentially spliced to generate three transcripts which code for the viral capsid proteins: VP1, VP2 and VP3.

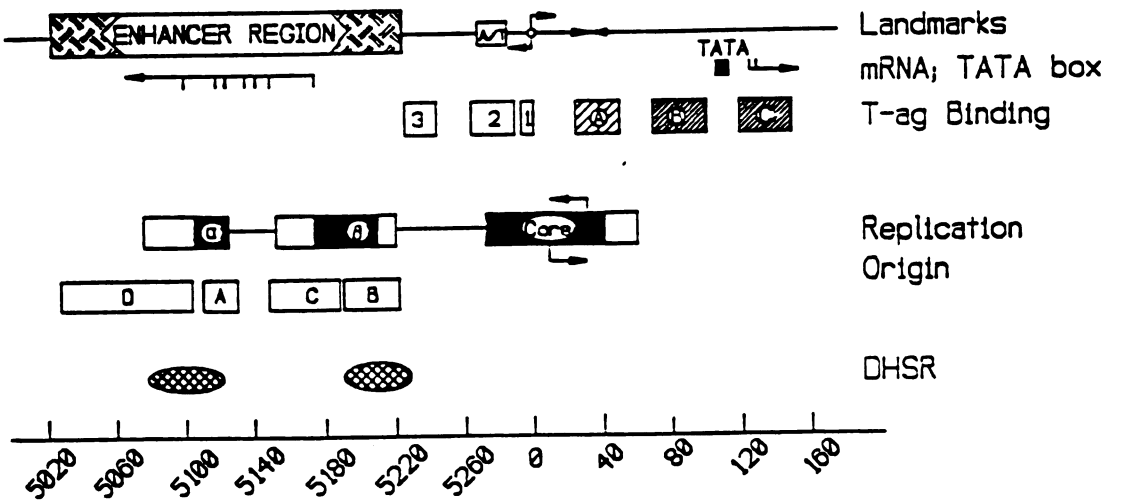
Studies of heteroduplex formation by electron microscopy (21), DNA hybridization (22), and immunological cross-reactivity (23,24) reveal extensive homology between polyomavirus and simian virus 40 (SV40). The primary homologous regions are the major viral capsid proteins, the VP1, and large T antigens. These data suggest that two viruses may have evolved from a common ancestor (25). However, three major regions of non-homology are present, including the noncoding regulatory regions, the region coding for the unique portion of middle T antigen in polyoma

genome, and the region encompassing the C-termini of both the large T antigen and the major viral capsid proteins.

All these regions are of unique importance, the first with its obvious regulatory effect on gene expression and replication, and two of the others in connection with cellular transformation by the individual virus. Since the two viruses interact with different permissive hosts, and since the activities of the regulatory region are dependent upon viral-cellular interactions, it is not surprising that this region contains different primary sequences. The major differences between polyomavirus and SV40 in the control region are in sequences defined as "enhancer" which have been demonstrated to be important for both viral DNA replication and gene expression in a host-specific manner (26,27). Distinct transformation mechanisms have been invoked by polyomavirus and SV40. Middle T antigen is the transforming protein of polyomavirus, which is located in the cell membrane and interacts with cellular kinases (28,29), and phosphatase 2A (30), leading to transformation of the host. By contrast, the transformation by SV40 is presumed to be due to the carboxy-terminus of its large T antigen binding to a cellular protein p53 (31). It has been suggested that both viruses may have diverged from a common ancestor and have acquired distinct properties to accommodate to their respective hosts during evolution (25).

Figure 2. Physical map of the noncoding region of the polyomavirus.

The center of the major palindrome in the origin of replication is (*ori*) defined as "0" and the nucleotide numbers extending from the late-gene side on the left to the early-gene side on the right are shown. The landmarks include the enhancer region, a 15-bp A/T sequence (open box), a 34-bp palindrome (pinwheel), a 17-bp inverted repeat (→←), a TATA box (solid box) and early and late mRNA start sites. DNA binding sites for large T antigen are shaded according to binding affinity (B,C > A > 1,2,3). *cis*-Acting sequences that function as the polyoma origin of replication (α,β and Core) are shown (shaded areas designate the minimum required sequence for these elements). The transition points from discontinuous to continuous DNA synthesis that define the origin of bi-directional DNA synthesis (OBR) are indicated by arrows protruding from *ori*-core. The four replicational enhancer domains (A-D) are shown in boxes. The DNase hypersensitive regions (DHSR) are shown in ellipses. The figure is summarized from Veldman et al. (34) and Hendrickson, et al. (91).


ENHANCER REGION
TATA
Landmarks
mRNA; TATA box
T-ag Binding
3
2
1
Core
Replication
Origin
D
A
C
B
DHSR
5020
5060
5100
5140
5180
5220
5260
0
40
80
120
160

Structure and Function of the Polyomavirus Enhancer

The noncoding region of the polyomavirus genome contains regulatory elements, including promoters for early and late gene transcription, origin sequences (*ori*) for initiation of DNA replication, and enhancer region (Fig.2) (2). "Enhancer elements" have been found in a variety of viral genomes and cellular genes (32). Generally, they act by stimulating transcription from linked polymerase II promoters in an orientation- and distance- independent fashion (33). The polyomavirus enhancer is required for both viral DNA replication, early and late gene transcription (34).

The polyoma enhancer is composed of two distinct non-overlapping enhancer elements, which are defined as the restriction fragments *Bcl*I-*Pvu*II (A or α enhancer, from nucleotides 5021 to 5132), and *Pvu*II-*Pvu*II (B or ß enhancer, from nucleotides 5133 to 5269) (35,36) (Fig.3). Although lacking appreciable homology, these two elements can substitute for each other in mouse fibroblasts to activate both early transcription and DNA replication (34,35). Extensive deletion mutagenesis and linker scanning analyses have further dissected the entire enhancer region into several structurally distinct domains that are functionally redundant. A and D domains are located in the restriction fragment *Bcl*I-*Pvu*II, B and C domains are in the fragment *Pvu*II-*Pvu*II (34). Each domain contains sequence motifs which

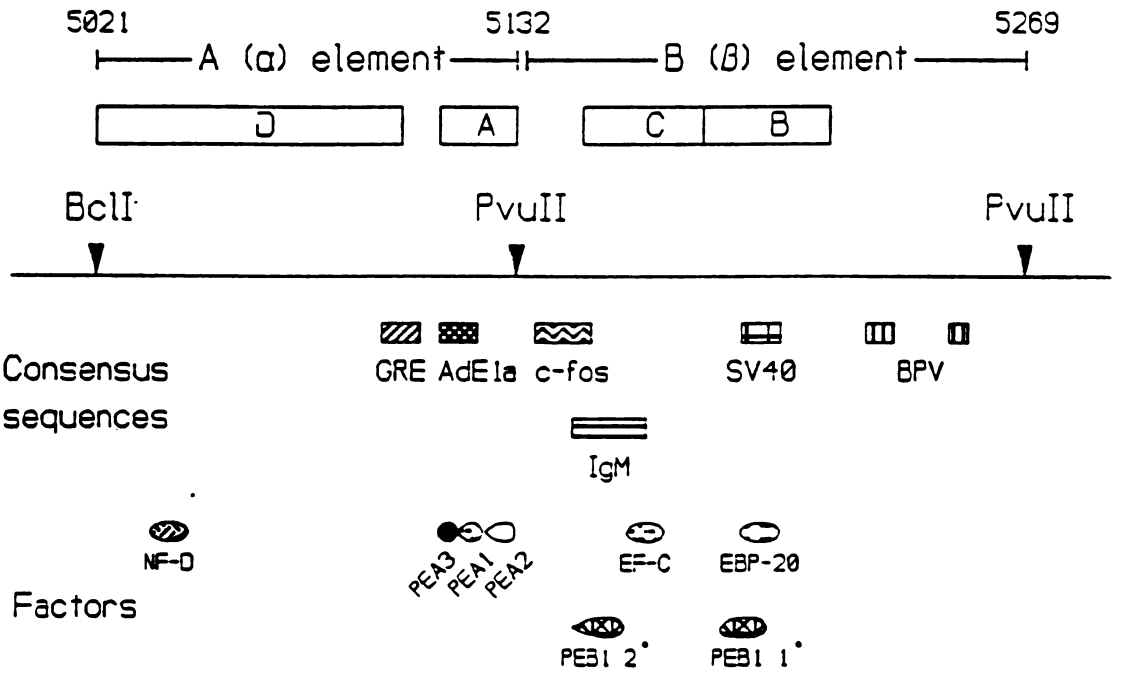


Figure 3. Cellular factors which bind to the enhancer region of polyomavirus.

The enhancer A (α) and B (β) elements, and the enhancer subdomains A, B, C, and D are indicated according to the restriction map of the polyoma enhancer. Regions of homology to sequences found in bovine papillomavirus (BPV), SV40, mouse immunoglobulin M (IgM), c-fos, adenovirus E1a enhancers, and glucocorticoid response element (GRE) are shown in boxes. Positions of cellular factor binding sites are depicted by ovals.

are homologous to several enhancer consensus sequences found in numerous other unrelated enhancers (Fig.3) (34).

Factors Which Interact with the Polyoma Enhancer

Cellular proteins have been shown to bind to enhancers in order to regulate transcription in a positive or a negative manner (37,38). Utilizing gel shift, DNase I footprinting, and methylation interference-protection assays, several of these transcription factors have been shown to bind to the polyoma enhancer (Fig.3). The interaction between the host protein and the enhancer sequence is tissue- and growth-state specific.

PEA1, PEA2 and PEA3 are three proteins from mouse nuclear extracts found to bind to the sequences within the A domain (nucleotides 5110-5132) (Fig.3) (39). These three nuclear factors recognize distinct neighboring sequences. PEA1 belongs to a closely-related family of transcription factors which includes human AP1, the product of the avian sarcoma virus v-*jun* oncogene and yeast GCN4 (40-42). Recently, products of the proto-oncogenes *ets*-1 and -2 have been shown to activate transcription through binding to the PEA3 motif (43). Cooperation of three oncoproteins c-Jun, c-Fos and $p68^{c\text{-}ets\text{-}1}$, therefore may activate from A domain of polyoma enhancer to the highest transcription efficiency. Both PEA1 and PEA3 but not PEA2 are able to mediate

transcriptional activation by serum growth factors, tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate) and several transforming oncogenes (44-46). PEA2 has been shown to act as a negative factor to repress the PEA1 activity in undifferentiated F9 EC cells, leading to a low activity of the A domain in these cells (47). In fibroblast cells and retinoic acid treated F9 cells, in contrast, a decrease in repression by PEA2 as well as an increase in the activity of a positive factor result in a large increase of A domain activity (47). The complex interactions of three transcription factors with the 23 nucleotides of the A domain suggest that a comparable balance must be involved in the cell response to growth stimuli.

The *Pvu*II-*Pvu*II fragment contains two functional enhancer domains, B and C, which interact with numerous cellular factors (Fig.3). PEB1 is a nuclear protein from murine cellular extracts interacting with the polyoma enhancer B domain. Its major binding sequences map to nucleotides 5184-5196, which encompass a region homologous to the early proximal portion of the GC-rich palindrome and the core sequences of SV40 enhancer (54-56). A nuclear factor isolated from rat liver, EBP20, strongly interact with sequences along nucleotides 5189-5199 within the B domain (57). The binding motifs of EBP20 and PEB1 overlap the SV40 core enhancer homology sequences. A minor PEB1

binding region (nucleotides 5146-5162) is located in the C domain, which partially overlaps the enhancer consensus sequences of c-*fos*. Nucleotides 5159-5175 within the C enhancer domain interacts with a nuclear factor, EF-C. The EF-C factor was initially identified in F9 cells but is also present in various other differentiated cell lines (58,59).

D domain is less well defined than the other three enhancer domains. Recently, a nuclear factor (NF-D) was found to bind to the nucleotides 5038-5045 within the polyoma enhancer D domain (Fig.3). A specific effect from the sequences comprising the binding site for NF-D has yet to be demonstrated, however, this factor is ubiquitous and comparably abundant in several murine cell types (61). Both the A and D domains have been shown to provide basal late promoter activity, and alteration of the NF-D binding site can decrease the late promoter activity (62).

Negatively acting transcription factors which interact with the enhancer sequences have also been described (47,54,56,65,66). The negative activity of the adenovirus 2 (Ad2) E1A proteins has been examined extensively. The E1A proteins are able to repress the transcription of a reporter gene which is linked to the polyoma enhancer (67,68). This inhibitory effect of the E1A proteins is also observed with AP1 factor-binding motifs from the SV40 and human metallothionein enhancers, and the MHC class I gene H-2K^b

enhancer, which binds the KBF1/H2TF1/TC-IIB protein (66). It is possible that the E1A proteins affect the activation of polyoma enhancer by inhibiting the activity of PEA1. Nuclear receptors (eg. the estrogen and glucocorticoid receptors), yeast enhancer factor GAL4 expressed in HeLa cells and chimeric trans-activators (such as GAL-VP16) are also similarly inhibited by the E1A gene products. It is therefore conceivable that the E1A proteins play a crucial role in repression by interfering in the transcriptional activation process at a step common to all *trans*-acting enhancer factors (69).

The polyoma enhancer is likely to have evolved in the context of the intricate cellular control mechanisms responsible for modulating gene expression. The presence of negative enhancer sequences seems contradictory to the presumed positive evolutionary selection. It could be because that the same enhancer sequences interact with either activators or repressors depending on different stages of cell differentiation. Alternatively, enhancer sequences may interact with a single cellular factor, whose activity is determined by the stage of cell differentiation (i.e., PEA2). Villarreal and colleagues have shown that individual polyoma enhancer elements display their own tissue specificity which is different from the wild-type pattern (86). Therefore, coexistence of several motifs

recognized by various cellular factors in the polyoma enhancer may provide a survival advantage through stages of cell differentiation and host development, and wide tropism in the mouse.

Altered Enhancer Regions in Polyomavirus Host-range Mutants

Reiterations in the polyoma enhancer have been found frequently among natural viral strains (TOR, P16, and CSP) (71). These sequence rearrangements appear to have a selective advantage in replication, resulting in the emergence of these viruses. Recently, much attention has been focused on the enhancer region of polyoma mutants that have a broader host range than do the wild-type strains.

Many host-range mutants have been isolated by selection for their ability to grow in undifferentiated mouse embryonal carcinoma (EC) cell lines. It appears that enhancer sequences in the B element are critical for growth of polyoma EC mutants in undifferentiated EC cell lines. Point mutations and tandem duplications of sequences around this region are always observed in the mutants isolated from F9 EC cells (48-51). By contrast, those isolated from infection of PCC4 cells have deletions in the B element and duplications in the A element (52,53). A variety of host-range mutants capable of growth in normally nonpermissive cell lines have been isolated. They include mutants which grow in trophoblastic, neuroblastic, or Friend erythro-

leukemic cell lines (63,64,72-74). Each type of mutant carries a distinct rearranged enhancer structure responsible for the respective host range. Rearranged enhancer elements can also strongly affect the ability of polyoma to replicate in mouse lymphoid cell lines (75). Therefore, the spectra of rearrangements in the enhancer region coincide with alterations of the host range.

Mutations within either the EBP20 or EF-C binding motifs has been shown to be detrimental to polyomavirus enhancer activity in F9 cells. In addition, a critical point mutation for productive infection of F9 cells is an AT to GC transition at nucleotide 5235 of the polyomavirus A2 genome. This mutation, located within the enhancer B element of the F9 EC mutant F441, creates a new positive acting sequence that can interact with a cellular factor in undifferentiated F9 cells (60). An endogenous "E1a like" activity has been proposed to exist in the undifferentiated EC cells. It is based upon the observation that the E1A products do not repress a point mutant (Py ECF9.1) of the polyoma enhancer which is active in the undifferentiated F9 cells (67). Moreover, a labile protein has been reported to prevent the replication of wild-type polyomavirus in undifferentiated PCC4 cells (70). The properties of the EC mutants implicate that alteration of the enhancer sequences responds to the qualitative or quantitative spectra of regulatory factors that exist within a particular cell type.

PyNB11/1 is a polyoma mutant selected for its high efficiency of growth in neuroblastoma cells (63). It has an altered enhancer region which contains a 91-base-pair tandem duplication spanning nucleotides 5047-5137 involving the A domain and part of the D domain (64). A novel site at the A/D junction of its enhancer region has been found to bind to a nuclear factor that belongs to the NF-1 family of transcription factors (61). It is thus suggested that new protein recognition sites in the polyomavirus enhancer may be generated by using modules which may pre-exist. Consequently, the new protein binding sites allow the host range flexibility in viral growth.

A cooperative action of enhancer components has also been implicated in tissue-specific replication. Replacement of the B domain with the Moloney murine leukemia virus enhancer drastically alters in vivo mouse organ infection patterns. This mutation redirects virus replication to the pancreas, which is normally nonpermissive to either of the parental viruses (76). An F9 mutant strain fPyF9 acquires three copies of cellular sequences (box DNA) which are inserted into the enhancer B domain. Box DNA functions as a negative transcriptional element in undifferentiated F9 cells but not in differentiated L cells, and allows the persistence of an episomal state of the viral genome in F9 EC cells (65,77). Comparing the enhancer sequences of these mutants, it is obvious that changes in specific components

of the enhancer region correlate with the ability of fPyF9 to propagate in specific cell lines.

In the development of this thesis, I will discuss novel alterations of enhancer discovered in host range transformation defective (hr-t) mutants of polyomavirus. These mutants were first isolated by Benjamin in 1970 using polyoma-transformed 3T3 cells as a permissive host and normal 3T3 cells as a nonpermissive host (78). The host range and nontransforming mutations coincide in these mutants, and are located in a viral protein coding region common for both small and middle T antigens (79,80). These mutants also contain differences in the noncoding regions comparing to the wild-types A2 and A3 (80,81). In the past, this has been assumed to reflect the organization of the wild-type from which they were derived. However, sequence analyses of the enhancer region of 7 hr-t strains show that each strain bears a unique alteration in this region (81, this study), which does not correspond to the wild-type. It is more likely that each sequence rearrangement in individual mutant has been evolved independently. A common feature to all the rearrangements involves the reiteration (1-3 times) of the A enhancer domain which contains middle T antigen responsive sequences, PEA1 and PEA3 (44,46). The theme which will be developed here is that the pathway of transformation or middle T antigen induced signal transduction is exquisitely coupled to the pathway for

polyoma enhancer activation. Thus, the duplication of the PEA1/PEA3 binding sites may represent a compensatory mutation for the absence of middle T and/or small T antigens, and confer a *cis*-advantage for viral growth during the isolation/selection.

Sequence rearrangements in the enhancer region which alter the spectra of factor binding, gene expression and viral replication also have been described in SV40, human polyoma virus (BK, JV). It is therefore suggested that papovaviruses may utilize this variability to adapt to various host cells and tissue types.

Cis-acting Sequences Which Control the Replication of Polyomavirus DNA

Cis-acting sequences that function as origins of DNA replication in polyomavirus genome contain two primary components: a core component (*ori*-core region), dedicated to DNA replication, that is required for replication under all conditions, and an auxiliary component, containing enhancer elements, that is involved in transcription as well as replication and is dispensable under some conditions.

The *ori*-core contains a 17-base-pair inverted repeat on the early gene side, multiple T-antigen binding sites within a GC-rich 32-base-pair sequence of dyad symmetry adjacent to a region in which 15 residues form A-T pairs (Fig.2). These

features are similar among polyomavirus, SV40 and BK virus, suggesting that secondary structures or symmetric binding is involved in the function of the origin (2). However, the *ori*-cores of polyomavirus and SV40 differ in several aspects. The major T-antigen binding sites in SV40 are superimposed with the replication origin, while the major polyoma T-antigen binding sites B and C lie outside of *ori*-core. The TATA box and cap-sites for SV40 early mRNA synthesis are superimposed on ori-core, while these elements in polyomavirus are outside of *ori*-core (Fig.2) (2). The fact that polyoma viral DNA replication requires a transcriptional enhancer may be attributed to its different genome structure from SV40's around the *ori*-core.

The enhancer region is required <u>in cis</u> for efficient synthesis of polyoma viral DNA (34,82). In mouse COP-5 cells, which constitutively express polyoma large T antigen, SV40 or immunoglobulin enhancers can replace polyoma enhancer to restore replication of a plasmid containing polyoma *ori* sequences (83). Further studies show that the dimeric ordered combination of PEA3 and PEA1 factor-binding sites represents the minimum sequences that are able to stimulate viral DNA replication to wild-type level (84,85). The perfect correlation between stimulation of DNA replication and those binding sites for transcription factors suggests that the enhancer function *per se* is

required for viral DNA replication and may not be specific to the sequences utilized. Unlike the transcription enhancement, the distance between the enhancer and the *ori* core may be critical in some but not all cells for replication activation (83,85,86). It is hypothesized that the closeness of the enhancer to the AT-rich end of *ori*-core might facilitate interaction between *ori*-core and proteins, thereby unwinding DNA templates and initiating DNA synthesis. These processes could be accomplished by either direct interaction of the enhancer binding proteins with *ori* binding proteins or modification of the chromatin structure around the *ori*-core by enhancer binding proteins (87). It has been shown that, in cell-free systems the polyoma *ori*-core functions without an enhancer (88). Furthermore, the polyoma *ori*-core sequence does not require an associated enhancer component to initiate replication until formation of two-cell mouse embryos (89). Perhaps some negative regulatory factors that interfere DNA replication are associated with the nuclear structure (i.e., chromatin structure, matrix, scaffold). The enhancer elements, when associated with its specific binding factors, release the origin from the nuclear matrix.

Analyses of initiation of DNA synthesis using various eukaryotic and prokaryotic model systems indicate that similar strategies are shared, which involve complex

formation of the *ori*-core sequence with initiation proteins (90). The initiation site of polyoma DNA synthesis has been mapped to a 16-base-pair position of the inverted repeat (91). The first step is the ATP-dependent binding of large T antigen to the specific sites, which leads to cascades of DNA conformation change (untwisting, and unwinding) (92-95). Production of underwound DNA appears to be a prerequisite for initiation of DNA synthesis. Two diverging replication forks are generated by the initiation complex. Large T antigen acts as a helicase translocating along the parental DNA strand allowing the entrance of other proteins (94, 100). DNA synthesis from the replication fork is semi-discontinuous, using host enzymes for primer synthesis (96), elongation (97,98), gap filling (99), ligation (2,9), etc.

The general features of polyoma viral DNA replication resemble those observed for SV40 DNA replication. However, it has been noticed that mouse cell lines support the replication of polyomavirus but not of SV40, whereas monkey and human cells replicate SV40 but not polyomavirus. The established cell-free replication systems provide a way to examine the cellular proteins involved in permissivity (10,101,102). SV40 DNA is replicated in the presence of the SV40 large T antigen and extracts from either human or monkey cells (103). In contrast, polyoma viral DNA replication requires the polyoma large T antigen and mouse

cell extracts (10). Addition of purified mouse DNA pol α/primase complex to HeLa or monkey cell extracts allows *in vitro* synthesis of polyoma viral DNA, suggesting that the DNA pol α/primase is a major factor in permissivity for papovaviruses. Monoclonal antibodies to SV40 large T antigen have been shown to co-precipitate large T antigen and DNA pol α from SV40 infected HeLa cell extracts, indicating that these proteins interact directly (104).

It is likely that cellular proteins other than pol α/primase are responsible for the permissivity of polyoma DNA replication. In rat cell lines, viral DNA synthesis is higher at 33°C than at 37°C, and only 1-2% of the polyomavirus transformed cells replicate the integrated viral genome to high level (105). Treatment of polyoma transformed rat embryo muscle cells (LPT line) with physical and chemical carcinogens or agents that inhibit protein synthesis can increase the percentage (≤0.2%→90%) of cell population producing large amounts of viral DNA (106). The permissivity factors in those systems remain to be determined. As discussed above, the varieties of enhancer binding factors in different mouse cell types affect permissivity of viral DNA replication; the hr-t mutants replicate less efficiently in normal mouse cells than in Py3T3 cells (78); wild-type polyoma strains fail to replicate in undifferentiated EC cells (48-53).

Large T antigens of polyoma and SV40 are the only virus-encoded proteins that are involved in viral DNA replication in cell-free systems (10,101,102). The function(s) of large T antigen in initiation have in fact shown considerable similarity to those of E. coli gene products, dnaA, dnaB, and dnaC, in initiating replication at ori C (90). Requirement of large T antigen for initiation of DNA synthesis was first shown by utilizing a viral mutant (ts-a). The thermolabile large T antigen of the ts-a strain fails to initiate new rounds of viral DNA replication at the nonpermissive temperature (106). Binding of large T antigen to the *ori* also results in negative autoregulation of early gene expression and induction of late gene expression (108-111). However, the overproduction of SV40 viral RNA in ts mutant infections is temperature independent, suggesting that the effects on RNA transcription and DNA replication are not coordinate (112). Although large T antigen stimulates transcription from cellular promoters (113,114), the DNA binding domain of large T antigen is not required for the activation (114).

Polyoma large T antigen is a nuclear phosphoprotein that contains 785 amino acids (Fig.4) (1). At the initiation step of polyoma viral DNA synthesis, large T antigen functions as a sequence specific binding protein (107-109), with ATPase (115), and helicase activities

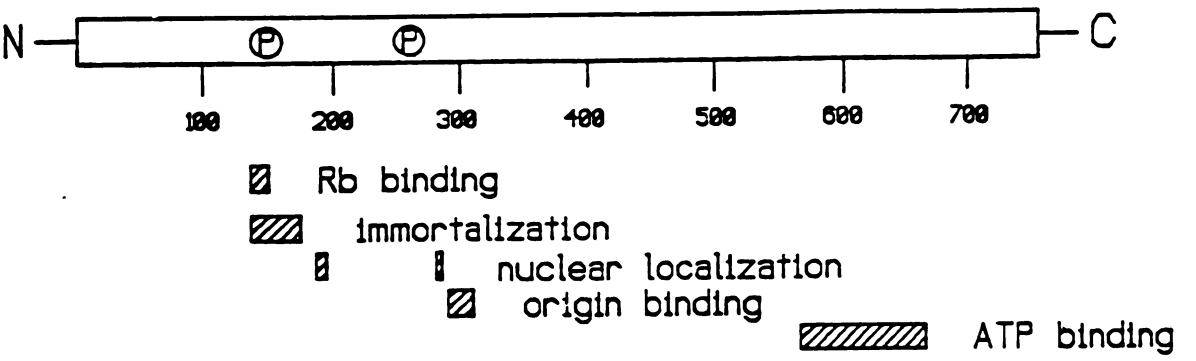


Figure 4. Schematic representation of phosphorylation sites and functional domains in the polyomavirus large T antigen.

Two main regions of phosphorylation sites are depicted by circles inside the large T antigen box. Below the T antigen are the approximate positions of domains related to its function.

(94,100,117). The nucleotide binding domain of the large T antigen has been mapped to a region between amino acids 290 and 310, whereas, the ATPase activity is located near the carboxy-terminus between amino acids 565 and 675 (116,117) (Fig.4). While acting as a DNA helicase, large T antigen moves in the 3'→5' direction on the DNA strand to which it is bound.

The phosphorylation state of large T antigen may play a critical role in its ability to perform multiple functions within the infected cells. Since large T antigen encoded by ts-a mutants is underphosphorylated (118) and is not able to initiate new round of viral DNA synthesis (106), the phosphorylation state may be crucial to viral DNA synthesis. McVey et al. have shown that cdc2 protein kinase phosphorylates the large T antigen of SV40, which results in an efficient binding of the large T antigen to the SV40 *ori* (119). Another protein that regulates the phosphorylation state of the SV40 large T antigen is protein phosphatase 2A (PP2A). PP2A removes serine phosphates from large T antigen, which reduces the lag time prior to new DNA chain synthesis and stimulates the T antigen- and SSB-dependent unwinding of DNA fragments containing the SV40 *ori* (120,121). Since the activities of cdc2 protein kinase and PP2A are both cell cycle regulated, the cell cycle stage of infected cells may affect the level of large T antigen

phosphorylation. It is consistent with the observation that the large T antigen of polyomavirus is more phosphorylated in the growing cells early in infection than in the quiescent cells (118). The cdc2 kinase and PP2A studies indicate that the replication-related phosphorylation of large T antigen may vary at different stages of the cell cycle. Therefore, it suggests that actively cycling cells rather than resting cells provide the optimal state to modify large T antigen to initiate viral DNA replication.

The other two early proteins, middle T and small T antigens play a major role in cell transformation as will be discussed below. Their potential roles in viral DNA replication have not been examined in detail. However, most studies using mutants with altered middle T and/or small T antigens suggest that these proteins are not directly required for viral DNA replication. Hr-t mutants, which are both middle and small T antigen defective, have been shown to display the following phenotypes: absence of secondary induction of c-*myc* and c-*fos* expression in host cells (8,125); decreased output of viral DNA (123,124); underacetylation of histone H3 and H4 in the viral minichromosome (122); underphosphorylation of VP1; and defects in the assembly of viral capsids into mature virions (123). Hr-t mutants stimulate only one round of cellular DNA synthesis in contrast to the wild-type strains which

stimulate multiple rounds (126). As discussed further below, middle T antigen has been well characterized for its role in oncogenic transformation, correlated with its interactions with cellular membrane proteins such as $pp60^{c\text{-}src}$ and phosphatidylinositol (PI) kinase (28,29). In the presence of small T antigen alone, NIH-3T3 cells reach a higher saturation density than untreated monolayers (127). These observations suggest that these two proteins act pleiotropically to alter the physiological state of the host in a manner which facilitates virus production in the lytic infection. Interestingly, these two proteins have been shown to associate with the regulatory subunits of PP2A (30,128). How these interactions affect the activities of large T antigen remains to be resolved.

Mechanisms of Cell Transformation by Polyomavirus

The early viral gene products of polyomavirus, the so called T antigens, were recognized originally by use of sera from tumor-bearing animals (129). Each of the T antigen species plays some role in the process of establishment or maintaining cell transformation. Complementation studies utilizing two groups of nontransforming mutants, hr-t and ts-a strains, determine the viral proteins involved in neoplastic transformation (12,130,131). As mentioned above, the major mutation of the hr-t strains has been mapped to

the intron of the large T antigen and affects both middle T and small T antigens (79,80). The ts-a mutants carry mutations at the carboxy-terminus of large T antigen (79,132). Therefore, the stable transformation caused by polyomavirus requires cooperative functions of large T antigen and either middle T antigen, small T antigen, or both middle T and small T antigens.

Genetic studies have shown that large T antigen is essential for the initiation of transformation (11). At the nonpermissive temperature, ts-a mutants are capable of inducing a transformed cell phenotype which lasts a couple of generations (abortive transformation), but are defective in stable transformation. However, ts-a mutants can transform cells at the permissive temperature, and a delayed shift to the nonpermissive temperature past the first two days does not affect transformation. The process of initiation is defined as events requiring transient expression of large T antigen and leading to the integration of viral genome into the cellular chromosome. Once the viral genome is integrated, large T antigen is no longer required.

Southern blot analyses of DNA from polyomavirus transformed cell lines have provided extensive knowledge regarding the arrangements of integrated viral genomes. The viral genomes are usually integrated in a head-to-tail tandem manner and inserted at multiple sites of the host

chromosome (133-135). Recombination between the host and viral DNA is non-homologous, with only 2-5 base pairs of partial homology at the viral host junction (136-139). Integration of polyomavirus can result in deletions, duplications, and rearrangement of host DNA at the integration site (139,140). There are no preferred sequences in the host genome found for polyomavirus integration (134,135,141). The variations in patterns among transformants imply that multiple independent integration sites are available in the host chromosome. In contrast, the observation of the double integration events from mixed infections of FR-3T3 cells are rare, which suggests that only a limited number of integration sites exist in the host chromosome (142).

Two models have been proposed for the integration of head-to-tail tandem viral genomes. Basilico and collaborators have shown that formation of integrated tandems requires a functional large T antigen (143) and viral origin of replication (143,144). It has been postulated that rolling-circle replication of the viral genome produces a linear multimer which may serve as a substrate for integration, consistent with a role for viral DNA replication. High molecular weight concatamers of viral genomes have been identified in infections of nonpermissive cells with SV40 (145). These have been hypothesized to represent precursors of the integrated tandem originating by

rolling-circle replication. However, a number of observations suggest that this is not the normal mechanism of how tandem genomes are generated. This mode of viral DNA replication represents a rare pathway in polyomavirus-infected mouse cells (146), and has not yet been documented in rat cells. Moreover, increased levels of DNA replication does not correlate with increased transformation frequency (105). Further evidence against this model come from "recombinant" tandem genomes. Recombinant tandems of viral genomes were first described in complementation experiments using ts-a and hr-t strains. Two parental genomes were found cointegrated at a single site in the host chromosome, suggesting that homologous recombination had generated a concatemeric structure as a precursor for the integrated tandem (147). In a system not selecting for recombination, a very high level of interviral recombination accompanying integration of the viral genome has been observed (148). At present, the data does not conclusively support either the replication or recombination model.

A recombinogenic activity for large T antigen has been implicated from several lines both with polyomavirus and SV40. Large T antigen is able to promote amplification and excision of integrated viral sequences as long homologies are provided (i.e., a repeated viral genome) (134,135,150). Large T antigen is required for resolving a polyomavirus-mouse hybrid replicon (149). A recent study by St-Onge et

al. shows that polyomavirus large T antigen promotes homologous recombination between viral sequences very efficiently, when the latter carry a functional origin of viral DNA replication (151). Direct evidence for the exact mechanism involved in the integration of polyomavirus genome concomitantly with neoplastic transformation is still missing. Possibly, it involves both recombination and replication of viral DNA.

Extrachromosomal forms of viral DNA (10-60 copies per transformed cell) are present in many transformed cells in addition to the integrated copies (152). Most of these free viral genomes are thought to result from excision of the integrated viral DNA following in situ replication (150,156). Excision requires homologous regions of polyoma sequence (153,154), large T antigen (135), and a functional origin of replication (155). It is likely that replication and recombination are involved in generating free copies of the viral genome. In situ amplification of integrated viral genome requires the same parameters as for excision (153,155). According to the "onionskin" model (156), initiation of replication at a given proviral locus leads to multiple rounds of DNA synthesis. The resulting structure of a localized onionskin with amplified sequences can then present a substrate for homologous recombination, leading to excision or amplification. Moreover, Basilico and coworkers have demonstrated that excision of integrated tandem repeats

can lead to the appearance of phenotypic revertants (134,135).

Another property of large T antigen concerns its ability to immortalize primary mouse and rat embryo cells (157). Immortalized cells do not display phenotypic changes associated with transformation, but usually a decrease in serum requirement is observed. The immortalizing function has been mapped to the amino-terminal portions of the large T antigen (Fig.4) (157,158). The mechanism of the large T antigen induced immortalization is not clear, but may be related to its action as a regulator of cellular gene transcription.

Middle T antigen is the major transforming protein of polyoma. This assertion stems back to the properties of hr-t mutants. Hr-t mutants, though making normal large T antigen and integrating into the cellular genome as a wild type, do not induce either abortive or stable transformation (159). Such findings support the argument that middle T and/or small T antigens maintain the transformation state. This was confirmed since plasmids which encode only middle T antigen are able to transform established rat cell lines (160,161) and are tumorigenic in newborn hamsters (162,163), whereas, transformation of primary rat embryo fibroblasts requires all three T antigens (161). In normal established rat fibroblasts, expression of middle T antigen under the control of a dexamethasone-inducible promoter elicits

various degrees of transformation correlating with levels of middle T antigen (168). Anchorage-independent growth (ability to grow in soft agar) and tumor formation require higher levels of expression than do focus formation and morphological changes (i.e., loss of actin cables and decreased adhesion). Mutations which affect the middle T antigen but not large T or small T antigen, can severely impair the ability of transformation (171).

As mentioned above, either small T or large T antigen has to be present to cooperate with middle T antigen to cause tumor in primary rat embryo fibroblasts (161-163). These observations support the theory that tumorigenesis is a complex process that requires multiple cellular events (164). Co-transfection of primary cells with proto-oncogenes and polyoma early genes provide further support for the theory. In the transformation of primary mouse embryo fibroblasts either large T antigen or c-*myc* can complement c-*ras* (165). Moreover, primary baby rat kidney cells can be transformed by a combination of the E1a proteins of adenovirus and either middle T antigen or c-*ras*. The large T antigen, c-*myc*, as well as E1a genes are identified as "immortalizing oncogenes" which are able to convert cell lines of limited replicative potential into ones which are capable of being passaged indefinitely. The middle T antigen gene belongs to the "transforming oncogene

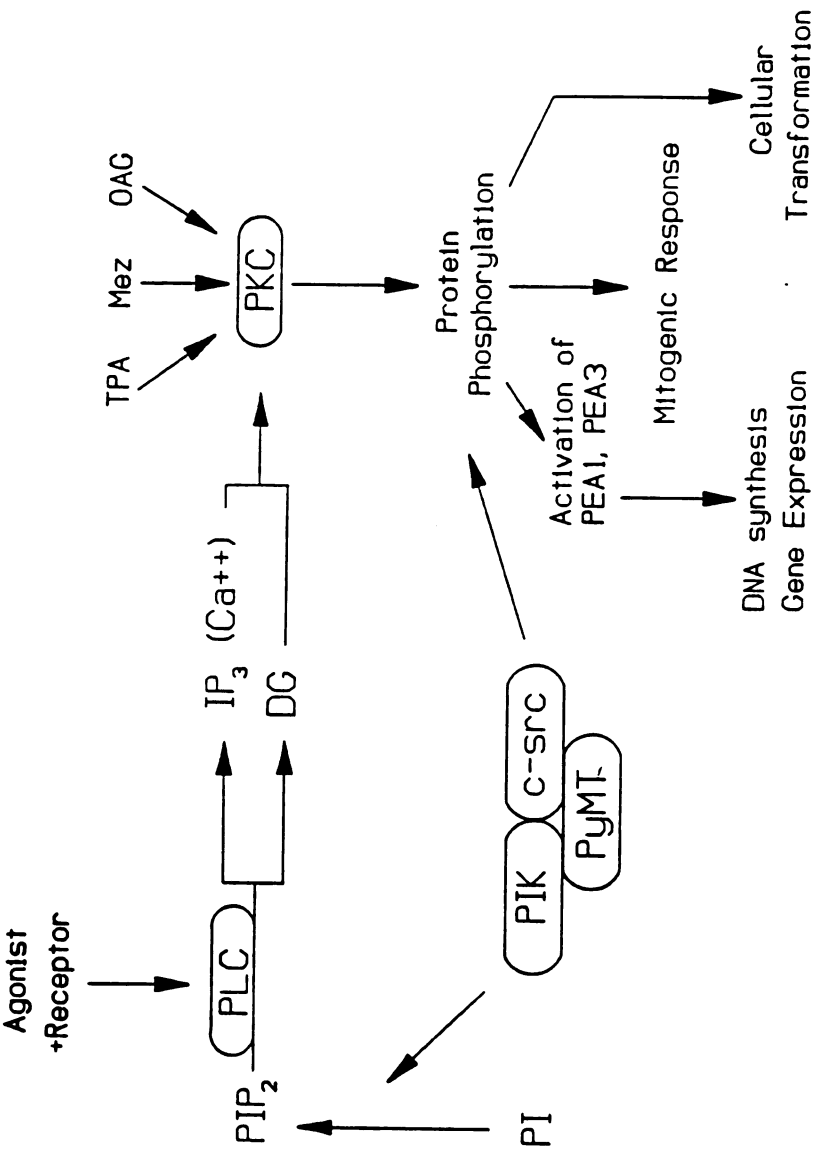
family" which confers anchorage-independent growth. The collaboration of both types of oncogenes prompts malignant transformation.

Middle T antigen is a membrane phosphoprotein of 421 amino acids, the amino-terminal 79 of which are identical to the amino termini of small T and large T antigens (167). Plasma-membrane associated middle T antigen is the major active fraction responsible for cell transformation (167-169). The protein is anchored in the membrane on the cytoplasmic side via a stretch of 20 hydrophobic amino acids near its carboxy terminus (170). Targeting the protein into the membrane is presumed to be a post-translational event since it lacks a signal peptide sequence at the amino terminus.

Although the middle T antigen has no known enzyme activities, at least three associated activities have been described. The major effects of middle T antigen come from its actions in regulating phosphorylation events in the cells. One of these is from its association with cellular tyrosine kinase of the src family (src, fyn, and yes, but not lck) (Fig.5) (28, 172-178). These three tyrosine kinases can be distinguished in that they are not identical in the way they bind to middle T antigen, with $p59^{fyn}$ showing a much lower affinity (28, 175-177). The association results in the activation of the tyrosine kinase

Figure 5. Model for the transforming action of polyomavirus middle T antigen.

Polyoma middle T, PyMT; Phosphoinositol kinase-3, PIK; cellular *src* proto-oncogene, c-src; phosphatidylinositol, PI; phosphatidyl inositol-bis-phosphate, PIP_2; inositol-tri phosphate, IP_3; diacyl glycerol, DG; phospholipase C, PLC; protein kinase C, PKC; 12-O-tetradecanoylphorbol 13-acetate, TPA; mezeriein, Mez; OAG, the diacylglycerol analogue 1-oleoyl-2-acetyl-glycerol; protein phosphorylation, PyMT/c-src-dependent tyrosine kinase activity and PKC-dependent serine/threonine kinase activity.


Agonist
+Receptor
PLC
PIP2
IP3
(Ca++)
DG
PI
PIK
c-src
PyMT
TPA
Mez
OAC
PKC
Protein
Phosphorylation
Activation of
PEA1, PEA3
Mitogenic Response
DNA synthesis
Gene Expression
Cellular
Transformation

activity. The complex formation between $pp60^{c\text{-}src}$ and middle T antigen has been studied most extensively. The kinase complex is associated with the plasma membrane (167,179, 180), and monoclonal antibodies to either middle T antigen or $pp60^{c\text{-}src}$ co-precipitate the two proteins from cell extracts of polyoma-infected cells (28). Only a small fraction of middle T antigen in an infected cell is associated with $pp60^{c\text{-}src}$ (174), and it seems to be the level of this complex which determine the transformation phenotype. The membrane association of $pp60^{c\text{-}src}$ depends on an amino-terminal myristate group covalently linked to glycine 1 of the mature protein (180,181). However, a nonmyristylated $pp60^{c\text{-}src(2A)}$ though failing to reside on the membrane, becomes membrane associated upon complex formation with middle T antigen with activation of the $pp60^{src}$ kinase activity (182). Therefore, the putative carboxy-terminus of middle T antigen is sufficient to hold the middle-T-$pp60^{c\text{-}src}$ complex in the plasma membrane.

The phosphorylation state of middle T antigen varies and influences the molecular weight of the protein. Middle T antigen has a molecular weight of 56-58 Kd depending on its state of phosphorylation (167,168). It is the 58 Kd form of the middle T antigen that associates and activates $pp60^{c\text{-}src}$. A nontransforming hr-t mutant strain, NG59, produces the 56 Kd phosphorylated middle T antigen which is able to associate with $pp60^{c\text{-}src}$ but is inactive in <u>in vitro</u>

kinase assay (185,186). In wild-type polyoma infections, TPA stimulates the phosphorylation of the 58 Kd form but not the 56 Kd one (187). Therefore, the increase of tyrosine kinase activity is likely determined by the phosphorylation state of the middle T antigen and $pp60^{c\text{-}src}$, which affects the interactions between the two proteins (183,185). By using site-directed mutagenesis and hr-t mutants, phosphorylation of the tyrosine residues on the carboxy-terminal half of the middle T antigen appears to affect the interaction between middle T antigen with $pp60^{c\text{-}src}$ which results in the formation of an active kinase complex (181,186,188). Genetic and mutagenesis analyses reveal that the amino-terminus of middle T antigen is also important for activation of the tyrosine kinase activity of $pp60^{c\text{-}src}$ and for transformation (189). Underphosphorylation at tyrosine 527 of $pp60^{c\text{-}src}$ is critical for the tyrosine-specific kinase activity (183). Phosphorylation at this position decreases the tyrosine-specific kinase activity of the protein (184).

Similar to mutated *src* protein, middle T antigen induces the signal transduction pathway mediated by phosphatidyl inositol (PI) kinase. Interestingly, however, the PI kinase involved in this pathway is a recently discovered enzyme which phosphorylates position 3 of the inositol ring to form phosphatidylinositol(3) phosphate [PtdIns(3)P] rather than the position 4 seen in the

phospholipase C-dependent signal transduction pathway (195). PI 3-kinase is a 81 Kd phosphoprotein which forms a stable complex with middle T antigen and all three activated forms of cellular tyrosine kinases (29,192,193), and is phosphorylated on tyrosine in the complex (29,194). Dephosphorylation of this protein either in vitro or during extraction has been correlated with the loss of its PI kinase activity. The presence of this PI kinase is most likely identical to the putative 85 Kd PI 3-kinase which binds to the PDGF receptor. The physiological functions of the PtdIns(3)P are not known, nevertheless, studies indicate that it might be important in mitogenesis and transformation (196-198). Genetic analysis has revealed a relatively close association between this PI 3-kinase activity and transformation. Its activity can be immune precipitated with all transforming middle T antigens and certain nontransforming middle T antigen mutants (29,192,199,200) but not with other nontransforming middle T antigen mutants which retain the ability to bind $pp60^{c\text{-}src}$ (190,191). Association of PI 3-kinase activity with the middle T antigen, therefore, appears necessary for transformation. But whether association is sufficient for cell transformation has not yet been resolved.

Although all middle T antigen mutants that lack associated in vitro tyrosine kinase activity are transformation negative, mutants which are transformation

negative yet are fully active in in vitro tyrosine kinase assay have been isolated. Substitution of tyrosine by phenylalanine at position 315 results in a mutant middle T antigen that activates $pp60^{c\text{-}src}$ but fails to serve as a substrate for $pp60^{c\text{-}src}$ and subsequently fails to bind to PI 3-kinase (170,190). This mutant middle T antigen transforms cells in culture weakly, and is decreased in the efficiency and spectrum of tumors in the newborn mouse. These data indicate that phosphorylation state of middle T antigen by $pp60^{c\text{-}src}$ is a critical event for binding of PI 3-kinase and for expression of the full tumorigenic potential of the virus. Furthermore, the observation of a similar elevated level of inositol triphosphate ($InsP_3$) in both mutant and wild-type infection suggests that association of middle T antigen with $pp60^{c\text{-}src}$ activates phospholipase C which is unrelated to the binding with PI 3-kinase (191).

Two proteins with molecular weights of 36 Kd and 63 Kd present in middle T antigen immunoprecipitates have been recently determined to be the catalytic and regulatory subunits of the serine- and threonine- specific protein phosphatase 2A (PP2A) (30,128). Again, genetic analysis suggests that the presence of these proteins is necessary but not sufficient for transformation by middle T antigen (201,202). However, this conclusion is based on data regarding the association of these two proteins with various middle T antigen mutants, rather than on measurements of

PP2A activity. PP2A is active in vitro against a number of enzymes involved in metabolic pathways and is thought to have an important role in the regulation of various metabolic processes (203). The precise physiological role for this enzyme in cellular transformation remains to be examined.

The 36 and 63 Kd proteins also bind to the small T antigens of polyomavirus and SV40 (30). These two proteins are the only proteins found associated with small T antigens in cell lysates. No mutant small T antigen, that lose function while retaining the ability to bind to these two proteins has been reported. It suggests that binding to PP2A may be sufficient for the small T function. The small T antigens cannot by themselves transform cells but are thought to act synergistically with the other T antigens to elicit various aspects of cell transformation (162,163). In particular instances, the small T antigen has mitogenic and cell-DNA-synthesis-stimulating activities for cells in monolayer culture (127,204,205). Through the interaction with PP2A, small T antigen may *trans*-activate certain promoters by modifying the activity of certain transcription factors (206).

Summary

From this literature review, it is obvious that a wealth of information about the polyomavirus has been accumulated over the past forty years. During the early years, most studies have been focused on the observation of biological interactions of the virus with permissive and nonpermissive hosts. The later progress made in molecular biology facilitates the elucidation of the intricate arrangement of the polyomavirus genome and of the biochemical changes which accompany viral infection. The discoveries of the alternative splicing of viral RNA and the replicational/ transcriptional enhancers shed light on control mechanisms of eukaryotic gene expression. Requirement of cellular enzymes for both viral DNA replication and RNA transcription provides a valuable model system for studying eukaryotic gene function. Each virally encoded T antigen plays some role in the process of establishing or maintaining cell transformation mainly by altering cellular properties. The primary role of these viral gene products are to promote virus growth. In the meantime, it provides an intriguing system for oncogenesis study.

In this study, effects of the polyomavirus enhancer on viral DNA synthesis and neoplastic transformation were investigated. Evidence for the existence of limiting factors for viral DNA replication in normal mouse cells was

compensatory event for the loss of middle T and/or small T antigens.

Enhancer mediated competition was also observed in nonpermissive FR-3T3 cells, suggesting that rat cells contain limiting enhancer factors. Wild-type virus induced stable transformation was interfered more pronounced than viral DNA replication and abortive transformation. Taken with the observation of low probability for double integration, we propose that integration is a rate limiting step during the process of polyomavirus mediated neoplastic transformation. It can be either due to the existence of limiting amounts of one or several factors required for integration step, or due to the limitation of integration events in the host chromosome. Since both viruses produce normal large T antigen, which is required for integration, it is likely that integration of one viral genome prevents a secondary event.

obtained. Competition assays revealed that the target sequences for these factors are located within the A enhancer element of the polyomavirus genome. In the absence of middle T antigen, a simple duplicated A enhancer element displayed growth advantage over the wild-type A2 enhancer. This phenomenon was alleviated in the presence of middle T antigen. Alleviation of enhancer competition was obtained by adding TPA and serum growth factors, suggesting that the middle T antigen affect enhancer activity on DNA replication through a transformation or signal transduction pathway. Given that all natural hr-t isolates have evolved with sequence rearrangements in the same region of the enhancer element, it is likely to be a compensatory event for the loss of middle T and/or small T antigens.

Enhancer mediated competition was also observed in nonpermissive rat cells, since transformation frequency of the wild-type virus was decreased by the hr-t mutants in a dosage- and enhancer context- dependent manner. The observation of low probability for double integration suggests that integration is a limiting step during the process of polyomavirus mediated neoplastic transformation. It can be either due to the existence of a limited amount of one or more of the factors required for integration step or the limitation of integration events in the host chromosome. Since both viruses produce normal large T antigen in the

infected cells, we propose that the competing virus prevents the integration of wild type virus.

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Chapter 2.

DNA Replication of Polyoma Variants with Altered Enhancers[1]

ABSTRACT

The DNA replication capacity of polyomavirus was studied in mixed infections of NIH-3T3 cells. The results show that viral genomes compete for cellular factor(s) present in limiting amounts. Studies of enhancer mutants suggest that nuclear factor(s) binding to the A enhancer element represent the major class of candidates. Kinetic studies imply that competition occurs and accumulates after the initial round(s) of DNA replication and that the limiting step is an initiation step. Furthermore, studies with hr-t mutants (lacking both middle and small T antigens) reveal that middle T and/or small T antigen play a major role in DNA synthesis, presumably by inducing factors which bind to the A enhancer.

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Introduction

Polyomaviruses such as polyoma (Py) and SV40 DNA have provided a useful model for the study of eukaryotic DNA synthesis (7). Efficient replication of the Py genome requires *cis*-acting sequences, virally encoded large T antigen, and cellular components from a permissive host (24,28). The *cis*-acting sequences, encompassing the non-coding region of the viral genome (35), include the origin-core and enhancer elements (6,8). The origin-core contains minimum sequences needed to replicate Py DNA in a cell free system (29,45). Large T antigen binds to sequences within and adjacent to the origin-core, and is required for the initiation of viral DNA synthesis (11,14,28). Once bound to the viral genome, large T antigen unwinds DNA around the replication origin, thereby permitting subsequent entrance of host cellular enzymes to proceed DNA replication.

The mechanism by which the enhancer sequences affect transcription and replication of Py DNA is under investigation. It has been shown that the enhancer plays dual roles in directing Py DNA replication as well as transcription in a cell-specific manner (6). Mutations in this region simultaneously alter both DNA replication and transcription in specific cell types, suggesting that the enhancer activates both processes through a common pathway (2,4,40). The minimal enhancer sequences required for full

levels of viral DNA replication varies from cell-type to cell-type (30,40). However, the enhancer is dispensable for DNA replication in vitro (29), and in the early development of mouse embryos at the one-cell stage (23). Therefore, it is possible that different cellular *trans*-acting factors, chromatin and nuclear structures lead to the tissue-, stage-, or cell-specificity.

Up to now, a number of enhancer binding proteins have been identified in different cell lines (5,12,18,22,25,26, 27). Some of these proteins interact in vitro with specific sequence motifs within the Py enhancer. In vivo studies demonstrate that binding of some of these *trans*-acting factors activates replication of the Py genome cooperatively (24,30). Mutation of certain factor binding sequences can abolish the replication in some cell lines. More interestingly, mutations may create novel factor recognizing sequences, and allow the virus to grow in a previously nonpermissive host (5,19,21,26,38). Natural variations in enhancer sequences have been reported among Py strains of similar as well as different host-range groups, but certain common features are observed within the same group (19,21,38,39). It appears that the rearrangements in the enhancer somehow modify the interaction between the viral genome and cellular factors, consequently leading to altered tissue tropism with regard to the ability to replicate and

transcribe in different hosts (2).

Host range transformation deficient (hr-t) mutants were selected as viable mutants of Py with altered host range and transformation deficiency shown to be mediated by mutations in middle T and small T antigens (9,37). As shown elsewhere, these mutants evolved with a uniquely rearranged enhancer compared to the wild-type strains A2 or A3 (1,16 and Chapter 3 in this thesis). In addition to other unique variations in the enhancer structures, sequences between nucleotides (nt) 5088 and 5146 are found reiterated two to four times in each of seven viral genomes analyzed. The region of overlap contains binding sites for 3 nuclear factors, PEA1, PEA2 and PEA3, of mouse cells (22). PEA 1 and PEA 3 represent the mouse analogs of the oncogenes *jun* and *ets*, respectively (27,41). They also function cooperatively to activate transcription and replication (30,42,43). In this article, we studied the effect of amplification of these oncogene-responsive transcription motifs on Py DNA replication and their biological significance in hr-t mutants.

Materials and Methods

Cells and viruses. Mouse NIH-3T3 cells were grown in 1X Dulbecco modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated newborn calf serum and maintained in a

humidified incubator with 5% CO_2 at 37°C.

All viral strains were grown on baby mouse kidney (BMK) cultures from plaque purified viruses (44). A2 strain is a large-plaque virus, used as a reference wild-type in our laboratory (15). Hr-t mutants were originally screened for their ability to grow on polyoma-transformed 3T3 cells (Py-3T3) and their failure to grow on normal mouse 3T3 cells (3). They have lesions in both small T and middle T antigens but none in large T antigen (33,34). The mutations have been mapped to *Msp*I restriction fragment #4 in the proximal part of the early region (9,16). A9, B2, 3B4, and 6B5 were isolated from lysate of a Pasadena small plaque strain mutagenized by the mutagen ICR-191 (37). Mutant II-5 was isolated from lysates of a Pasadena small plaque virus after two high multiplicity passages on Py-3T3 cells (37). 18-5 is an hr-t mutant constructed by site directed mutagenesis from wild-type A2, and contains an approximately 50 bp deletion in the *Msp*I fragment #4 (20).

Enhancer exchanges. Viral DNA from A2- or B2- infected NIH-3T3 cells were prepared by the method of Hirt (17). Viral DNA was digested with restriction enzymes *Bcl*I and *Bgl*I, and each *Bcl*I-*Bgl*I fragment was gel-purified. The 375 base-pair (bp) *Bcl*I-*Bgl*I fragment of mutant strain B2 was ligated to the 4954 bp *Bcl*I-*Bgl*I fragment of wild-type A2. A total of 50 ng of viral DNA fragments were ligated in 10 μl

of ligation buffer (66 mM Tris, pH 7.5; 10 mM $MgCl_2$; 1 mM DTT; and 1 mM ATP) for 16 hours at 16°C using T4 DNA ligase (New England Biolab). For transfection, 10 ng of the ligation mixture was applied to $1.5x10^5$ NIH-3T3 cells/35 mm dish using 500 μg/ml DEAE-dextran at 33°C for 1 hour. The dishes were rinsed with 1X Tris-buffered saline and serum free 1X DMEM, overlayed with DMEM containing 0.9% agar and 5% newborn calf serum, and incubated at 37°C. Plaques were picked, and viral stocks were grown on BMK or NIH-3T3 cells. The resulted recombinant virus is named as A2(eB2), which is transformation-competent as strain A2 and contains a B2 enhancer-origin region.

Infections. NIH-3T3 cells were seeded at a density of $2x10^5$ per 60 mm plate and were infected after attachment. The relative ratio of two viral genomes in the infection mixture was established from the titers of the viral stocks, and confirmed from inputs by hybridization analysis of low molecular weight DNA extracted from infected cells at 4 hours post infection. Cells were fed with 1X DMEM supplemented with 2% heat-inactivated newborn calf serum after infection.

Analysis of viral DNA. Low-molecular-weight DNA was sampled by the method of Hirt (17) at the designated time points post infection. After phenol/chloroform extraction and 95% ethanol precipitation, the DNA was suspended in 0.05 ml of 10 mM Tris hydrochloride (pH 7.4)-1 mM EDTA ($T_{10}E_1$).

For analysis, unless otherwise indicated, 1/10 volume of each sample was digested with *Msp*I, electrophoresed on a 2% Borate-Tris agarose gel, and hybridized with a specific probe after Southern transfer to nitrocellulose (36).

Preparation of DNA from lysates of infected cells. Infected cell monolayers and supernatants were harvested at 5 days post infection when the cells showed signs of lysis. Lysates were sonicated for 1.5 minutes to release the virus from cell debris. Total lysate DNA was isolated by digestion of 0.4 ml of lysate with 50 μg/ml proteinase K and 0.5% SDS at 37°C overnight. After phenol/chloroform extraction, DNA was precipitated with 0.3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol. The DNA was resuspended in $T_{10}E_1$ as described above. To obtain encapsidated viral DNA, we removed free DNA from the lysates by treatment with 50 ng/ml DNase I in the presence of 10 mM magnesium chloride at 37°C for 2 hours. DNase I was inactivated by adding 15 mM EDTA and 0.2% SDS. The resulted mixture was then treated with proteinase K and SDS to purify virion DNA. For analysis, one-tenth volume of total lysate DNA and one-third of the virion DNA were treated as described above.

Hybridization analyses. ^{32}P-labeled pPy-1, representing the complete polyomavirus genome in pBR322, or ^{32}P-labeled pG4PyH4, representing the *Msp*I-4 in pGem 4

plasmid (Promega), was used as probe in DNA hybridizations to Southern blots at a specific activity of $1x10^9$ to $6x10^9$ cpm/μg. Hybridizations were carried out in 1x Denhardt's solution-2x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate)(0.1 ml/cm_2) at 65°C for 24 hours with $0.5x10^6$ cpm/ml probe. Comparisons of the intensities between DNA bands in the same blot were determined by densitometric scanning.

Results

Quantitative analysis of viral DNA synthesis by competition assay. As reviewed in the Introduction, all the factors and enzymes required for replication of Py DNA are derived from the host cell, except for large T antigen (7). To study the capacity of the host replication machinery, we designed a competition assay. NIH-3T3 cells were infected with a mixture of wild-type strain A2 and a viable deletion mutant strain of the hr-t type (18-5), and the levels of accumulated viral DNA were analyzed. In each infection mixture, we varied the input ratio between parental strains by keeping the multiplicity of A2 at 1 and varying the multiplicity of 18-5 from 1 to 10. Input ratios were verified by hybridization analysis of DNA extracted four hours post infection (Data not shown). Low molecular weight DNA was extracted at 60 hours post infection and analyzed by *Msp*I digestion, which differentiated the two viruses by the

fragment #4 (Material and Methods). Fig.1 indicates that as the multiplicity of 18-5 is increased from 0 to 10 in the input, a 20% decrease in the yield of wild-type A2 DNA is observed. This result implies that limiting amounts of replication factors are present in NIH-3T3 cells. Since both parental strains produce normal large T antigen (33,34), it is likely that the limiting factor is of cellular origin.

At the MOI ratio of 1 to 1, we found that deletion mutant 18-5 replicated less efficiently than the wild-type A2. We think this could have resulted from an effect of low multiplicity. Since at the MOI of 1, approximately 30% of the cell population received both parental viruses and most infected cells contained either A2 or 18-5. Most signals in the extracts, therefore, represent accumulated DNA from singly infected cells. The result of underreplication of 18-5 in this infection is consistent with our observation that this virus grows poorly (see Discussion).

Effect of the enhancer configuration on the competition for DNA replication. Given the precedent that binding of nuclear factors to the enhancer A domain is required for Py DNA replication and transcription (22), and reiterations in the enhancer A domain provide a *cis*-advantage for growing in several types of mouse cells (21), we investigated the

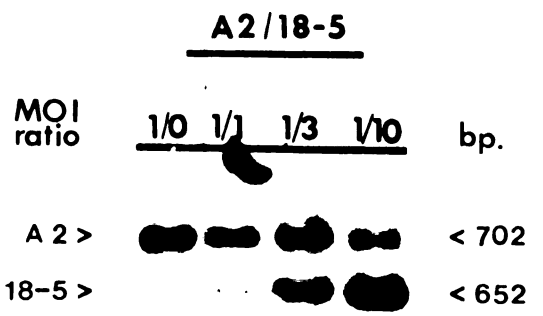


Figure 1. Replication of A2 virus in competition with deletion mutant 18-5.

NIH-3T3 cells were infected with viral mixtures A2 and 18-5 at the MOI ratios of 1:0, 1:1, 1:3, and 1:10. Low molecular weight DNA was isolated at 60 hours post infection. One tenth of the sample was digested with restriction endonuclease *Msp*I, electrophoresed on a 2% agarose gel, transferred to nitrocellulose, and hybridized with a polyoma virus probe specific to *Msp*I restriction fragment #4. The upper band of 702 bp represents the *Msp*I fragment #4 of A2, and the lower band of 652 bp corresponds to the *Msp*I fragment #4 of 18-5.

effect of the enhancer duplication on the competition for viral DNA synthesis. For this purpose, we made use of an hr-t strain B2, which contains a 42 bp tandem duplication (nt 5103-5144) that encompasses the A domain when compared to wild-type A2 (Chapter 3). We also constructed a recombinant wild-type virus A2(eB2), whose noncoding regulatory region was replaced with that of B2 (Materials and Methods). We carried out competition assays by mix infecting NIH-3T3 cells with wild-type strain, A2 or A2(eB2), and a deletion strain B2. Accumulated viral DNA was analyzed as described above. We found that when the input MOI of the B2 strain was increased up to 10, DNA replication of wild-type A2 and A2(eB2) was decreased 83% and 30%, compared to that obtained in the single infections (Fig.2). However, the level of competition was less dramatic in the mixed infections with A2 plus 18-5, and A2(eB2) plus B2 than in that with A2 plus B2. The dosage-dependence of the competition supports our previous conclusion that NIH-3T3 mouse cells contain limiting amounts of replication factors. Moreover, the difference in the extent of competition influenced by different enhancer structures suggests that one of the target(s) of limiting factors is present on the duplicated A enhancer sequences.

Effect of other hr-t mutants on A2 replication in the mixed infections. To generalize the effect observed in the experiment using strain B2 as a competitor, a variety of

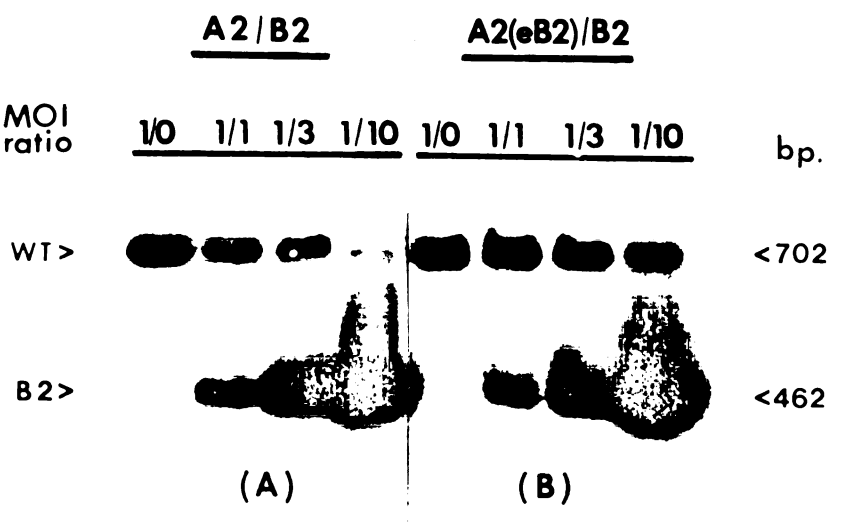


Figure 2. Competition assay in the mixed infections with various wild-types and deletion mutant B2.

NIH-3T3 cells were doubly infected with viral mixtures A2 and B2 (A), A2(eB2) and B2 (B), at the MOI ratios of 1:0, 1:1, 1:3, and 1:10. Viral DNA was analyzed as described in Fig.1. The size of *Msp*I fragment #4 for the wild-types is 702 bp, and for strain B2 is 462 bp.

other hr-t mutants were tested. As shown in Fig.3, using A2 and hr-t deletion mutants at a ratio of 1 to 10, all these hr-t mutants suppressed the replication of A2 DNA. When compared to the mixed infection with A2 derived hr-t strain 18-5 (lane 6, Fig.3), naturally isolated hr-t strains appear to be stronger competitors than 18-5. Furthermore, the magnitudes of reduction of A2 DNA varied amidst different infections. Strain A9, which contains 3-4 copies of A enhancer sequences (1, Chapter 3), induced the strongest competition (lane 2, Fig.3).

Competition in the absence of middle T antigen. The A domain of the Py enhancer, which contains binding sites for factors PEA1 and PEA3 (22), appears to be the target of the competition. Since both PEA1 and PEA3 are presumed to be activated by middle T antigen (42), it became of interest to study the effect of middle T antigen on the competition. An example is shown in Fig.4. In this experiment, NIH-3T3 cells were doubly infected with middle T antigen deficient strains B2 and 18-5. The multiplicities and the ratios of two viruses are indicated in Fig.4. Accumulated viral DNA extracted at 60 hours post infection was analyzed with *Msp*I, since the two parental strains contain different sizes of deletions in fragment #4. Compared to experiments with wild-type (Panel A, Fig.2), competition by B2 was more intense in the absence of middle T antigen. For example, comparing the output of 18-5 at the MOI ratio of 1 to 1,

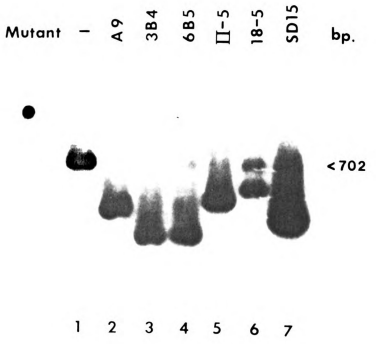


Figure 3. Effect of hr-t mutants on A2 replication.

NIH-3T3 cells were infected with a mixture of A2 and an hr-t deletion mutant at the MOI ratio of 1:10. The strain of hr-t mutant used is indicated above each lane. Viral DNA was analyzed as described in Fig.1. Output of A2 viral DNA in the mixed infection (lanes 2-7) is compared to that from the single infection with A2 virus (lane 1). The band of 702 bp represents the fragment #4 of A2, and lower band in each lane is the fragment #4 of the respective hr-t mutant used in the mixed infection.

we found a 50% and 93% decrease at the ratio of 1 to 3 and 1 to 10, respectively (Compare lanes 1-3, Fig.4). In contrast, 18-5 did not exert a strong competitive effect on B2 (lanes 4 and 5, Fig.4), suggesting the existence of *cis*-advantage growth of B2 over 18-5.

In fact, a significant competition was clearly observed at the relative ratio of 1:1, when the MOI was increased from 1 to 10 (lane 6, Fig.4). Less discrepancy between the two viruses at the MOI of 1 could be due to more of a population than a competition effect, and to the replication incompetence of 18-5. This is consistent with the previous interpretation that B2 replicates more efficiently than does 18-5.

Kinetic analysis of the competition event. To examine the kinetics of competition, the time course of viral DNA replication in a mixed infection with A2 and B2 was followed. Fig.5 shows the analyses of low molecular weight DNA collected at 4, 24, 48, and 72 hours post infection. To get an adequate picture of the results, we applied various amounts of DNA sample (see Figure legend), and analyzed samples of the two earlier time points (4 and 24 hours) hours separately from those of the later time points (48 and 72 hours). We observed that the onset of viral DNA synthesis occurred before 24 hours post infection (Compare Fig.5A and 5B). At 24 hours post infection, A2 DNA

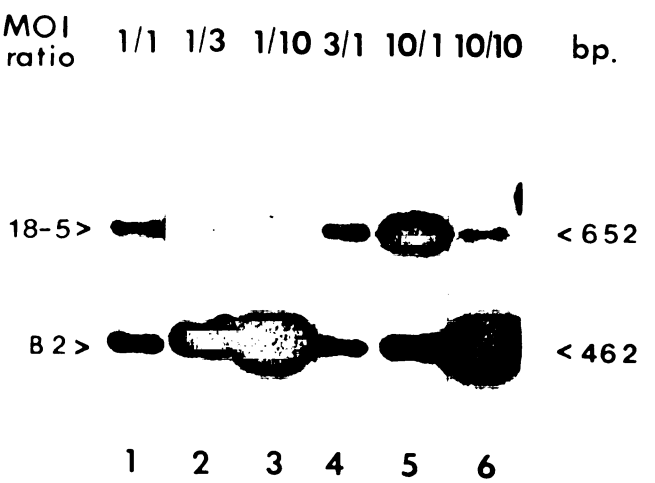


Figure 4. Competition for DNA synthesis between middle T antigen-deficient mutants.

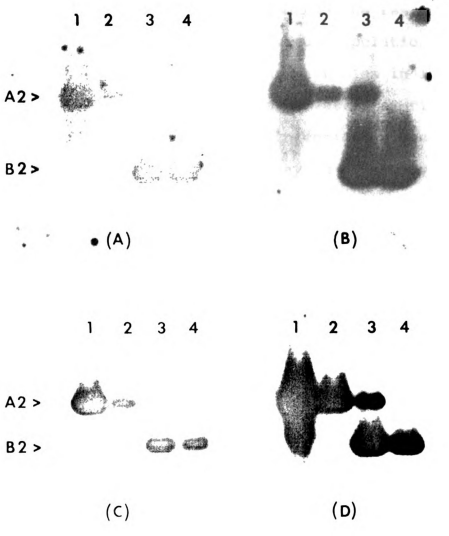
NIH-3T3 cells were doubly infected with strains 18-5 and B2. The MOI ratio of 18-5 to B2 is indicated above each lane. Accumulated viral DNA was isolated and analyzed as described in Fig.1. The bands of 652 and 462 bp are the fragments #4 of strains 18-5 and B2, respectively.

replication at the MOI of 1 reached the same level in both single and mixed infections (Fig.5B), and the ratio between A2 and B2 genomes maintained the same as that in the input. Competition occurred between 24 and 48 hours post infection, since less A2 DNA accumulated in the mixed infections than in the single infections (Compare lanes 2 to 3, Fig.5C and 5D). Whereas, the level of B2 DNA replication reached the same level in both single and mixed infections (Compare lanes 3 to 4, Fig.5C and 5D). Competition resulted in lower ratios of A2 to B2 than that in the input (Compare lanes 3 in Fig.5C and 5D to 5A and 5B). The repression of A2 DNA synthesis in the mixed infection suggests that not every A2 genome entered rounds of replication after 24 hours post infection.

When comparing the level of DNA accumulation in those single infections using A2 virus, we found that the yield obtained from the MOI of 1 never reach the same level as that from the MOI of 10 (at least till 72 hours post infection) (Compare lanes 1 to 2 in Fig.5). The increased capacity of DNA synthesis at a higher multiplicity can be attributed to an increase in the number of infected cells and mitogenic effect induced by capsid attachment (46). Moreover, the ratios of accumulated DNA between the two infections were kept constant throughout the time course. This implies that only a fixed portion of DNA molecules are in the replication pool.

Figure 5. Kinetics of viral DNA synthesis.

NIH-3T3 cells were infected with wild-type A2 alone at the MOI of 10 (lanes 1), and 1 (lanes 2); A2 and B2 at the MOI ratio of 1:10 (lanes 3), or with B2 alone at the MOI of 10 (lanes 4). Viral DNA isolated at 4 (A), 24 (B), 48 (C), and 72 (D) hours post infection was analyzed as described in Fig.1, except for various amounts of samples was used. The relative volumes used were one-half, one-fourth, one-tenth from the sample of 4, 24, 48 and 72 hours post infection, respectively.


1 2 3 4
A2 >
B2 >
(A)
1 2 3 4
(B)
1 2 3 4
A2 >
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(C)
1 2 3 4
(D)

Effect of competition on virus production. We also analyzed viral DNA accumulated in the lysates from mixed infections. Competition was also observed; for instance, less A2 DNA replicated in the presence of excess amounts of B2 (Fig.6A). To analyze whether and how the replication competition is represented in the viral population, we quantitated the yield of infectious particles in lysates from doubly infected NIH-3T3 cells harvested at five days post infection. Concentration of infectious virus in the lysate was determined by plaque assay on NIH-3T3 cells. Since this cell line is nonpermissive for hr-t mutants (3), the titer obtained from this assay would represent mainly the concentration of wild-type A2 virus in the lysate. Results shown in Table 1 indicate a dosage dependent decrease of wild-type virus yield in the presence of mutant virus. We also analyzed encapsidated viral DNA in the same lysates by treating the lysate with DNaseI prior to extracting virion DNA (Materials and Methods). Such DNA was then digested with *Msp*I to determine the ratio of the two parents. Results showed a consistency with that from the plaque assays, in which less encapsidated A2 DNA existed in the lysate from mixed infections (Fig.6B). Moreover, the magnitude of the decrease in virus production corresponded to that in encapsidated A2 DNA. The fact that the ratio of A2 to B2 are similar in virions and in the whole lysate

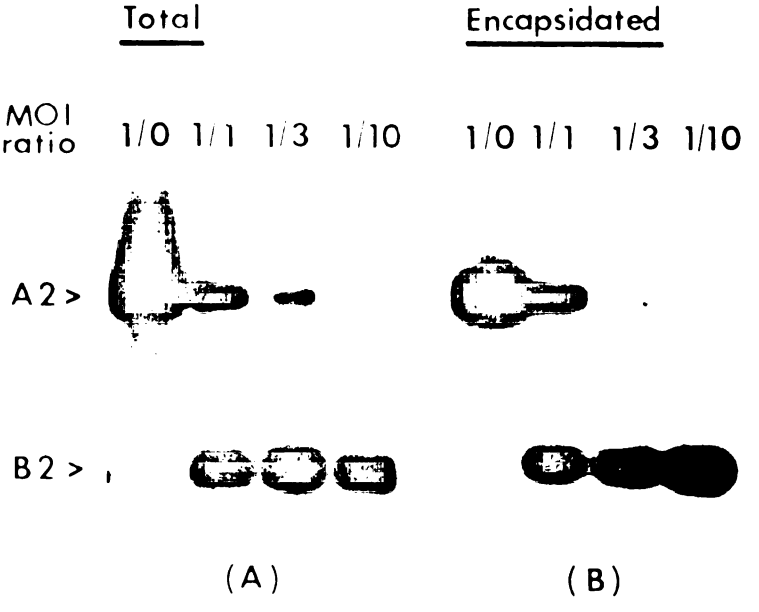

Figure 6. Accumulation of viral DNA in virions and whole lysate.

Viral lysates from A2 and B2 mixed infected NIH-3T3 cells were collected at 5 days post infection. Ratios of A2 to B2 in the infection mixture are given. Total (A) and encapsidated (B) viral DNAs in each lysate were isolated as described in Materials and Methods, and analyzed as described in Fig.1.

Table 1. Yield of infectious virus from mixed infections.

Viral strain		MOI[c]	Yield[d]
WT[a]	mutant[b]	WT:mutant	pfu/ml (x 10^5)
A2	B2	1:0	135
		1:1	102
		1:3	15
		1:10	1
A2	18-5	1:1	109
		1:3	80
		1:10	6
A2(eB2)	B2	1:0	174
		1:1	112
		1:3	60
		1:10	6

[a,b]Strains of middle T-proficient[a] and middle T-deletion mutant[b] used in the mixed infection.

[c]Ratio of multiplicities between the parental viruses in the infection.

[d]Summary of viral yields in lysates collected from mixed infections. Concentration of infectious virus was determined by plaque assay on NIH-3T3 cells.

(Compare Fig. 6A to 6B) implies that there is no preference of packaging either A2 or B2 DNA in the double infection.

Discussion

Polyomavirus relies mainly on the host's proteins and factors for DNA replication (7). The activities of these cellular components are believed to be well regulated during cell growth in the cell cycle, cellular differentiation, and cellular transformation. However, Py is able to bypass the cell-cycle regulation and reinitiates several rounds of DNA replication during the interval when host DNA has replicated only once. The experiments reported above were designed to investigate the ability of a Py genome to replicate in the host cell. For this purpose, we measured the DNA replication of a wild-type strain in NIH-3T3 cells in the presence of various amounts of a viable deletion mutant which was used as a competitor. Accumulated DNA of the two viruses was analyzed by restriction endonuclease digestion followed by electrophoresis which resolved the two parental genomes. The overall patterns of wild-type DNA yield derived from such infections indicate that NIH-3T3 cells have a limited capacity for Py replication, since the wild-type DNA was decreased by the mutant strain in a dosage dependent manner.

We also found that different competitor strains inhibited DNA replication of the same wild-type to various

degrees depending on the context of enhancer sequences of each mutant. Compared to the wild-type, strong competitors contain reiterations of the A domain (1, Chapter 3), suggesting that one of the targets for the competing factors is located in the A domain of Py enhancer. It also implies that a Py strain containing multiple copies of the A enhancer domain has *cis*-advantage for DNA replication.

All of the rearranged enhancer sequences tested in this study are derived from naturally isolated hr-t mutants. A previous report has described a "dominant lethal effect" for this group of Py mutants, in which hr-t strains exert a growth inhibition effect on wild-type strains (10). Our current studies suggest that competition for the A enhancer binding factors accounts for (at least part of) the "dominant lethal effect". Hr-t mutants have been selected for their transformation deficiency, and are able to grow in Py-transformed mouse 3T3 cells but not in normal mouse fibroblasts (3). A viral function expressed during transformation is thus proposed to be a prerequisite for productive infection of hr-t mutants. The defects of these viruses arise from their lack of middle T and small T antigens (9,16). Other variations in the regulatory regions of hr-t mutants have been noticed before (16), and detailed sequence analyses of seven of the hr-t strains demonstrate reiterations in the A enhancer domain (1, Chapter 3). The

commonality of reiterated A enhancer domain found among hr-t mutants leads to a speculation that this has been a compensation (co-evolution) event for viruses which lose their transformation function.

Duplications in the A enhancer domain have been found in several middle T antigen-proficient strains of Py (21,32). When compared to a strain with single copy of the A domain, these viruses display little *cis*-advantage for growth in normal mouse fibroblasts (21, Chapter 3). In contrast, we show that hr-t strain with sequence repeats has strong *cis*-advantage for viral DNA replication. It suggests that lack of middle T and/or small T antigens casts a selection pressure against those hr-t strains with single copy of the A domain. In fact, we have made efforts to grow up a chimeric hr-t mutant B2(eA2) which contains coding sequences from strain B2 and the noncoding region of wild-type A2. The difficulties in raising this particular virus and in preparing high-titer viral stock of strain 18-5 support this assumption. Such might be the case if middle T or small T antigen activate viral DNA synthesis by inducing the cellular factors that specifically bind to sequences in the A enhancer domain.

PEA1, PEA2 and PEA3 are three characterized, distinct nuclear proteins that bind to the A enhancer (22). The fact that middle T antigen induces Py replication by mediating

the activities of PEA1 and PEA3 (43) links the function of this transforming protein to a role in viral DNA replication. Furthermore, it has been suggested that hr-t gene induces a permissive state for Py growth in normal mouse cell, which can be bypassed in some primary cells or cells transformed by other viruses (37). Whether the amount of PEA1 and PEA3 in the host cells is one of the determinants of permissivity (or host range) for Py DNA replication in normal mouse cell lines will be further discussed in Chapter 3.

Py regulatory DNA includes an *ori*-core for replication and enhancer sequences. The organization of regulatory sequences in the viral genome, and the interactions of these sequences with virally encoded large T antigen and a variety of cellular factors are responsible for initiating viral DNA replication as well as for viral adaptability (2,8). The mechanism by which the enhancer activates DNA replication remains unclear, it is thought that binding of factors to the enhancer may prevent nucleosome assembly and allow formation of a initiation complex at the origin region of the viral genome. Thus we speculate that, in the presence of limiting amounts of nuclear factors, only those viral progeny DNA capable of binding to enhancer factors effectively proceed to subsequent rounds of DNA synthesis. In competition experiments, competitor strains with

reiterated factor binding sites strongly interfered the replication of wild-type DNA. This suggests that amplified A enhancer sequences in the mutant genome sequester PEA1 and PEA3 factors, thus reducing the number of available factor for the wild-type enhancer and leading to failure to form a replication complex at the origin. Radiolabelling experiments have shown that the majority of Py progeny are removed from the replicating pool following their initial synthesis, suggesting the existence of a rate limiting step for initiation (31). Consistent with this, our kinetic studies of competition showed that a greater proportion of replicated molecules reenter the replication pool at early rather than at late time post infection. In both single and mixed infections A2 DNA replicated to the same level till 24 hours post infection, whereas the level of A2 DNA was lower in the mixed infections during later courses of the infection. Possibly, as the number of total viral DNA molecules exceeded the amounts of PEA1 and PEA3 factors, and competition between viral genomes limited the accessibility of enhancer factors for A2 genomes in later rounds of DNA replication.

Results of competition for DNA replication were reflected in the output of viral particles, since the yield of infectious wild-type virus was depressed in the presence of mutants. The ratio between two viral genomes in the virions was the same as that in the whole lysate, which

suggests that the different enhancer structure did not affect encapsidation. Furthermore, we found that in both single and mixed infections only a portion (~30%) of viral genomes were effectively encapsidated, which can be due to the underphosphorylation of capsid proteins and inefficient viral assembly. It has been reported that middle T antigen plays an important role in maturation of the Py virion by phosphorylating capsid protein VP1 (13). In the mixed infection, the level of middle T antigen expression could be affected by two events: (1) competition for PEA1 and PEA3 results in competition for transcription, and (2) outcompeted wild-type DNA provides fewer templates for transcription. In analyses of virion DNA and plaque assays, a sharp decrease of A2 was readily observed at higher input ratio (1 to 3 rather than 1 to 10), which might result from the synergistic effects of the above two factors.

In conclusion, this report suggests that normal mouse 3T3 cells have a limited capacity for replication of viral genomes. One of the limiting host factors appears to interact with enhancer A domain of the Py genome, since strong competition can be observed for viruses with reiterations in this region. Middle T antigen may play an essential role in viral DNA replication by inducing the activities of those A enhancer binding factors, thus expanding cellular capacity to facilitate viral growth. The

mechanism of how a factor-enhancer complex is involved in the initiation of DNA synthesis remains to be understood.

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Chapter 3.

Functional Interdependence between Middle T Antigen and the Polyoma A Enhancer Region[1]

ABSTRACT

We show that hr-t middle T antigen deficient mutants which arose naturally and were selected for transformation dependent growth, have evolved with rearrangements in the enhancer. The variety of the rearrangements strongly suggests that each one of them represents an independent event. The rearrangements have one common feature, i.e., the reiteration of the A enhancer domain. This imparts a *cis*-advantage for replication to any strain (wild type or middle T antigen deficient) which harbors them. In the absence of the reiteration, middle T antigen deficient mutants have a strong defect in viral DNA replication. The previously reported "dominant lethal effect" of hr-t mutants in mixed infections with wild type can thus be explained as an enhancer-mediated competition effect. When two strains only differ by a duplication of the A element, the strong *cis*-advantage of the duplication carrying strain can be relieved partially by TPA or serum, and totally by middle T

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antigen. This suggests that these three agents, whose common targets are PEA1 and PEA3 motifs in the A enhancer element, act via the same or similar mechanism(s). The experiments suggest that the middle T antigen is much more effective than TPA or serum in inducing viral DNA synthesis via the activation of PEA1 and PEA3.

Introduction

Hr-t mutants are a group of host range transformation-defective mutants of polyomavirus (Py), which grow well on Py transformed mouse 3T3 (Py-3T3) cells but poorly on normal NIH-3T3 cells (2). They were isolated based on the assumption that integrated Py genes essential for viral growth would complement mutant strains defective in the homologous function. A variety of cell types were later found to be permissive for the growth of hr-t mutant without being transformed by Py. These include certain primary mouse cell lines, and some retrovirus-infected or -transformed 3T3 cells (15,42). Thus, it was hypothesized that efficient growth is achieved via gene activation of a permissive state in the host cell by the hr-t gene product(s), and that permissivity related cellular genes can be expressed constitutively or regulated independently of Py in some cell types (16). Whether the permissive state in non-Py transformed cells involves the same mechanism as that stimulated by the hr-t gene product(s) remain undetermined.

Cells infected with hr-t mutants lack certain traits induced by wild type infection, i.e., the ability to undergo multiple rounds of cell DNA synthesis, lectin agglutinability, and cellular transformation (16,38). The hr-t mutation has been mapped to the early gene coding region (11,18) and shown to alter the expression of small T and middle T antigens (37,39). Small T antigen, whose

function is still not very clear, has been shown to be involved in productive infection and anchorage-independent growth of transformed cells (6,26,31,46). Middle T antigen is the major transforming protein of Py (45), which associates with tyrosine kinases of the src family and phosphatidylinositol (PI) 3-kinase in the plasma membrane (3,5,7,24,44). Signals induced at the membrane are further translocated into the cytoplasm and the nucleus. In the cytoplasm, middle T antigen induces the phosphorylation of several proteins, including VP-1 (14) and the oncoprotein C-Raf (28). VP-1 phosphorylation appears to be essential for efficient encapsidation and assembly of infectious particles (13). Phosphorylation of C-Raf stimulates serine/threonine kinase activity of c-Raf, which may generate a cascade of signal transduction in the cell (28). Middle T antigen also enhances gene expression by activating cellular transcription factors PEA1 and PEA3 (49,50), which are the mouse homologs of human AP1 and c-ets, respectively (27,48). These two factors mediate transcription by binding to specific sequences located in the A enhancer element of Py (27). PEA1 and PEA3 mediated transcription is also stimulated by other oncogene products, TPA, and serum (22,53). Collectively, the activities of middle T (and small T) antigen alter the cellular transcription capacity, inducing the S phase of the cell cycle which is required for viral growth.

Hr-t mutants have been known to exert an inhibitory effect on virus yield in mixed infection with wild type (12). We recently observed that hr-t mutants interfere with wild type at the level of viral DNA synthesis (Chapter 2). This dominant lethal effect conferred by hr-t mutant is due to competition for enhancer binding factors present in limiting amounts. It has been noted that hr-t mutants differ from the prototypic strains A2 and A3 in the enhancer region (18). The Py enhancer has been divided into two domains, A and B, either of which can activate viral DNA transcription and replication in cis (47). Each element has distinct binding sequences for different cellular *trans*-acting factors (29,47). Different cell types demonstrate dependence on various enhancer subdomains to reach high levels of viral DNA synthesis, suggesting that each binding motif contributes significantly to DNA replication (4,20,35). The A enhancer element has been shown to activate transcription in response to TPA and six transforming oncogene products (22,49,53). TPA also stimulates the replication of DNA molecules containing the A enhancer and the Py origin (30). Both TPA and expression of middle T antigen affect protein kinase C activity and alter the physiological state of the host. Therefore, it is likely that middle T antigen enhances viral DNA replication by activating PEA1 and PEA3.

In this report, we have sequenced the enhancer alterations in hr-t mutants. We demonstrate a selective advantage for replicating virus with a duplicated A enhancer element. The selective pressure was accentuated in the absence of middle T antigen, in that a middle T deficient mutant containing the duplication strongly outcompeted a middle T deficient strain with single copy of the A enhancer element. The competition could be relieved by introducing TPA, serum, or middle T antigen. The results suggest that amplification of the A enhancer element complements the loss of middle T antigen in hr-t mutants.

Materials and Methods

Cells and viruses. Mouse NIH-3T3 cells (23) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated newborn calf serum and grown in a humidified incubator with 5% CO_2 at 37°C. Kidneys were removed from approximately 10-day old random-bred Balb/C mice and used to prepare primary cultures of baby mouse kidney (BMK) cells (52). BMK cells were grown in DMEM supplemented with 10% horse serum.

All viral strains were grown from plaque purified viruses on BMK cultures. Strain A2 is a large-plaque virus which is used as a reference wild type in our laboratory (17). When restriction endonuclease patterns of hr-t mutants were established (18), it became evident that these

mutants were derived from at least two parental strains, A and B. Both wild types A and B are of a small plaque phenotype. Wild type A harbors an 11 base-pair (bp) deletion of one of the large T antigen binding sites. Hr-t mutants NG-18, NG-59, HA-33, 3A-3, B2, 3B-2, 3B-4, and 30'b belong to this group. The other parental strain (B), which bears three large T antigen binding sites, harbors an additional *Hpa*II site in the late coding region resulting in a split *Hpa*II fragment 1. Hr-t strains NG-23, A2, A8, A9, A11, 6B5, SD-15, and II-5 are representatives of this pattern. Mutants NG-18 (Group A) and NG-23 (Group B) were derived at the same time from treatment with nitrosoguanidine, while strain HA-33 (Group A) was prepared from virus treated with hydroxylamine (2). Mutants A2, A8, A9, A11, and 6B-5 (Group B), as well as 3A3, B2, 3B-2, and 3B-4 (Group A) were derived in a later experiment from wild types treated with ICR-191 (42). Furthermore, mutant II-5 (Group B) was isolated from a wild type lysate after two high multiplicity passages on Py-3T3 cells (42). Mutants SD-15 (Group B) and 30'b (Group A) were isolated from the light side of a band of wild type virus in a cesium chloride equilibrium density gradient (42). Middle T antigen deficient strain 18-5 is derived from wild type strain A2 by deleting approximately 50 bp at 83 map units in the *Msp*I fragment #4 (25).

Enhancer exchanges. Viral DNA was prepared from infected NIH-3T3 cells by the method of Hirt (21). Viral DNA was digested with the restriction enzymes *BclI* and *BglI*. This generates two fragments: a small (approximately 350 bp) fragment containing the noncoding region including the enhancer, and a large (approximately 4,950 bp) fragment containing the coding region (See Fig.2). The two fragments were exchanged between various pairs of parental viral genomes. For this purpose, digested DNA was resolved by agarose gel electrophoresis, and each *BclI-BglI* fragment was excised. A total of 50 ng of viral DNA fragments were ligated in 10 μl of ligation buffer (66 mM Tris, pH 7.5; 10 mM $MgCl_2$; 1 mM DTT; and 1 mM ATP) for 16 hours at 16°C using T4 DNA ligase (New England Biolab). For transfection, 10 ng of DNA was applied to $1.5x10^5$ NIH-3T3 cells/35 mm dish using 500 μg/ml DEAE dextran at 33°C for 1 hour. The dishes were washed and overlayed with DMEM containing 0.9% agar and 5% newborn calf serum. Plaques were picked after staining with neutral red, and viral stocks were grown on NIH-3T3 cells.

The names of the resulting viruses consist of the name of the parental virus contributing the fragment with coding sequences followed in parentheses by the name of the virus contributing the fragment with enhancer region. For instance, A2(eB2) is an A2 virus in which the enhancer-origin region has been replaced with the corresponding

region from the B2 virus.

Sequence analysis of the noncoding region. Hirt DNA from each of the recombinant viruses was isolated from infected NIH-3T3 mouse cells and digested with *Bcl*I and *Pst*I to release the enhancer containing 600-700 bp fragment. This fragment was purified by low melting temperature agarose electrophoresis and ligated into the polylinker region of either the bacteriophage M13mp18 or M13mp19 RF DNA digested with *BamH*I and *Pst*I. Insert containing clones were isolated, and single stranded DNA of each was subjected to Sanger dideoxy sequencing from opposite directions using either the M13 universal primer or a 17mer on the early side of the polyoma *Bgl*I site.

Infections. Cells plated at a density of $2x10^5$ cells/60 mm dish were either singly or doubly infected with viral strains at a multiplicity of infection (MOI) of 1 or 10 plaque forming units (PFU)/cell. The relative ratio of the 2 parental genomes in the infection mix was originally established from the titer of the 2 parental viral stocks, and further confirmed by hybridization analysis of the viral sequences extracted from the cells at 4 hours post infection. Cells were fed with DMEM supplemented with newborn calf or horse serum as described in the text. TPA (Sigma Chemical Co.) was dissolved and diluted with ethanol and 10-100 nM was added into the incubation medium of each

dish.

Analysis of viral DNA. Low molecular weight DNA was extracted using the Hirt procedure (21), extracted with phenol and chloroform, precipitated with isopropyl alcohol, and resuspended in 50 µl of 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA. The DNA was digested with restriction endonuclease *Msp*I, which cuts polyoma DNA 8 times (18). The size differences in the enhancer region between viral strains can be distinguished by different mobility of the *Msp*I fragment 3 (18). The restriction fragments were separated on 1.8-2.0% agarose gels in 0.089 M Tris-borate, and 0.002 M EDTA (pH 8.0) and transferred to nitrocellulose or hybond membranes (Amersham Corp.) according to the Southern procedure (41). The DNA was fixed onto the blots and probed to a viral DNA fragment which specifically hybridized to the *Msp*I fragment 3. The polyoma *Msp*I-3 restriction fragment was isolated by *Hind*III and *EcoR*I digestions of a pGem plasmid containing this subfragment (which was originally inserted into the *Cla*I site in a pBR322 plasmid, and recloned into the *Hind*III, *EcoR*I sites of the pGem vector), and resolved in low melting point agarose (FMC Bioproducts). The probes were labeled to a specific activity of 1-2 x 10^9 cpm/µg [^{32}p]-dCTP (3,000 Ci/mmole) using a multiprime DNA labeling kit (Amersham Corp.). Hybridization was carried out at 65°C for 1 day in 2X SSC/1X Denhardt's or in 5X SSPE /5X

Denhardt's/0.5% SDS solution (0.1 ml/cm_2 with 1-2 x 10^5 cpm/ml of the labeled probes in hybridization solution).

RESULTS

Effect of hr-t mutants on A2 replication in mixed infections. We have recently demonstrated that in mixed infections of NIH-3T3 cells, replication of wild-type strain A2 is inhibited by increasing the amount of an hr-t mutant strain B2. The competitive effect of hr-t mutant B2 was shown to be greater than that of a middle T antigen defective mutant isolated by mutagenesis without the biological selection used for hr-t mutants (Chapter 2). This interference effect had been noted before (12) and referred to as "dominant lethal effect". To examine whether the "dominant lethal effect" exerted by strain B2 on wild-type viral DNA synthesis is a trait common to all hr-t mutants, we carried out mixed infections using wild type A2 and 15 hr-t mutants. In these, A2 was added at a multiplicity of 1, and the hr-t parent at a multiplicity of 10. Viral DNA extracted at 60 hours post infection was analyzed by *Msp*I digestion. Except for HA-33, all other hr-t mutants we used contain deletions of variable sizes in *Msp*I fragment 4 (18). In all cases, the level of A2 DNA was decreased in the mixed infections compared to the single infection with A2 alone (Fig.1). The output ratio

Figure 1. Effect of coinfection with hr-t mutants on A2 viral DNA synthesis.

NIH-3T3 cells were infected at a multiplicity of 1 PFU/cell of A2 with (lanes 1-15) or without (lane 16) an hr-t mutant which was used at a multiplicity of 10 PFU/cell. At 60 hours post infection, low molecular weight DNA was isolated. One tenth of the sample was digested with *Msp*I, electrophoresed on a 1.8% agarose gel, transferred to nitrocellulose, and hybridized to a probe specific to the polyoma *Msp*I restriction fragment #4.

hr-t
NG18
A2
A11
3A3
3B2
3B4
6B5
30 b
HA33
B2
NG23
A8
A9
11-5
SD-15
1
A2>
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

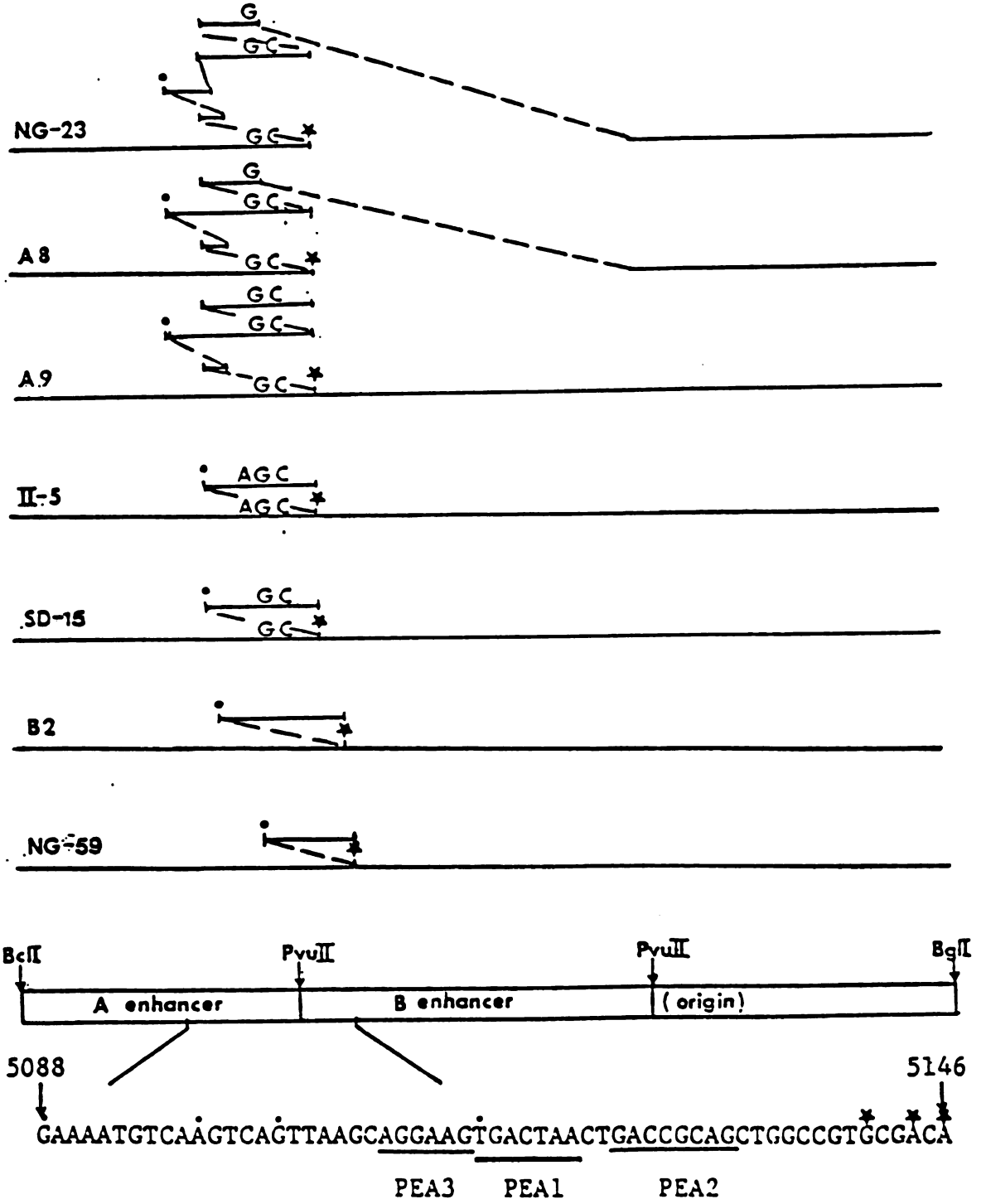
between A2 and mutant DNA varied with each mixed infection. In most cases except with NG-18, the band corresponding to wild type DNA cannot be detected, while bands corresponding to mutant DNA are approximately of similar intensity in all infections. This suggests that each hr-t mutant displays a certain extent of dominant lethal effect for viral DNA synthesis.

Sequence analysis of the enhancer region of hr-t mutants. Since the dominant lethal effect of hr-t mutant B2 is associated with the enhancer region of this virus, which contains a duplication (Chapter 2), we set out to sequence another five hr-t mutants between the *BclI* and the *BglI* sites. The results are illustrated schematically in Fig.2. The recently derived sequence of B2 as well as the sequences of NG-59 obtained by Ruley and Fried (36) are also shown for comparison. The sequence of NG-59 and B2 differ from the other five hr-t strains in that both contain an 11 bp deletion encompassing one of the major large T antigen binding sites (Data not shown). This variation has been reported between the prototype strains A2 and A3 (40).

Compared to wild types A2 and A3, all seven hr-t mutants display rearrangements in their enhancer regions. Strains NG-59 and B2 contain simple duplications of 31 bp and 42 bp, respectively. SD-15 and II-5 contain 44 bp

Figure 2. Comparison of enhancer-origin sequence of hr-t mutants.

The sequence of each of the hr-t strains is compared to a restriction map of the A2 strain of Salzman (8). The sequence of NG-59 is taken from Ruley, et al (36). The strand equivalent to the early mRNA is presented. Parallel lines represent duplications and dashed lines indicate their relation to the genome. Nucleotide substitutions are placed directly above their locations. Duplicated sequences between nucleotides 5088 and 5146 are shown at the bottom, leftward (·) and rightward (*) boundaries of each reiteration in the hr-t strain are indicated above the corresponding nucleotide. Nuclear factors PEA3, PEA1, and PEA2 binding regions within the A enhancer domain are shown.


NG-23
A8
A9
II-5
SD-15
B2
NG-59
AGC
BclII
PvuII
PvuII
BglII
A enhancer
B enhancer
(origin)
5088
5146
GAAAATGTCAAGTCAGTTAAGCAGGAAGTGACTAACTGACCGCAGCTGGCCGTGCGACA
PEA3
PEA1
PEA2

duplications identical to those described previously for the P16 and Toronto strains (36). The three remaining viruses have complicated patterns of rearrangement, with multiple and more extensive reiterations of the region between nucleotides 5088 and 5141. Interestingly, many of the rearrangements have an identical border. The rightward border of the duplication at nucleotide 5141 is shared by mutants NG-23, A8, A9, II-5, and SD-15. The leftward border at nucleotide 5088 is shared by NG-23, A8, and A9. Strains II-5 and SD-15 have the same leftward border at nucleotide 5098. Moreover, in strains A8 and NG-23 this amplification is coupled with a deletion of 123 bp between nucleotides 5141 and 5264. This deletion encompasses the B enhancer element of the A2 genome. Only SD-15 and II-5 have the same rearrangement (except for a point mutation in II-5). All other five strains differ from each other and from that of SD-15/II-5.

Though each virus contains a unique alteration, all have a common feature involving reiteration of the A enhancer domain. As described in the Materials and Methods, the seven hr-t mutants characterized here have different histories. Mutants NG-23, A8, A9, SD-15, and II-5 belong to the Group B. Though isolated at different time, they share a duplication between nucleotides 5098 and 5141. Thus, it appears that various and independent duplications of the A

enhancer domain arose in at least two different parental strains and were retained in hr-t mutants.

The effect of reiterated A enhancer elements on viral DNA synthesis. The enhancers (*BclI-BglI* fragment) of six hr-t mutants were introduced one at a time into the A2 genome (see Materials and Methods). These recombinants are designated A2(eX) where X refers to the origin of the enhancer fragment. To investigate whether the altered enhancer sequences affect viral DNA replication, competition experiments were performed in which pairs of viruses were used at a multiplicity of 10 for each. These viruses can be distinguished from each other by the size of their enhancer, for example *MspI* fragment 3. To verify the equal dosage of the two parents in the input, viral DNA was extracted at four hours post infection, digested with *MspI*, electrophoresed, blotted and hybridized with *MspI* fragment 3 as described in Materials and Methods (Data not shown). A mild advantage in DNA replication was observed for the enhancer B2 in competition with A2 (lane 1, Fig.3). Strain A2(eA9) which contains a triplicated A element outcompetes strain A2(eB2) with a duplicated A element (lane 4, Fig.3), and displays extensive growth advantage over the A2 strain (lane 3, Fig.3). These results confirm our observation that NIH-3T3 cells contain limiting factors for DNA replication whose targets are located in the A enhancer element (Chapter 2).

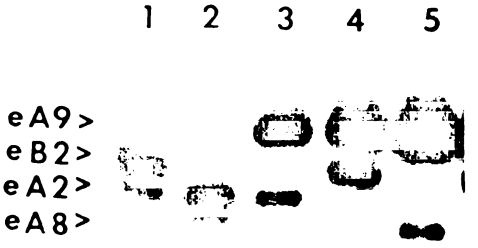


Figure 3. Effect of reiterated A enhancer element on viral DNA synthesis.

Recombinant viruses were generated by replacing the enhancer containing *Bcl*I-*Bgl*I fragment of the strain A2 with that of an hr-t strain. NIH-3T3 cells were infected at a multiplicity of 10 PFU/cell of each of the following viruses: A2 and A2(eB2) (lane 1), A2 and A2(eA8) (lane 2), A2 and A2(eA9) (lane 3), A2(eA9) and A2(eB2) (lane 4), A2(eA9) and A2(eA8) (lane 5). Viral DNA was analyzed as described in the legend to Fig.1, except that a probe specific to the polyoma *Msp*I-3 was used in the hybridization.

Though containing a triplicated A enhancer element, strain A2(eA8) displayed the lowest replication efficiency among the four strains tested (lanes 2 and 5, Fig.3). This implies that either a function of the B domain in viral DNA synthesis cannot be compensated by reiterations of the A domain, or that a site is created for a repressor in the A rearrangement. Given the similarities between the A8, A9, and NG-23 rearrangements, the latter is not very likely. The activities of the A and B elements of the Py enhancer in DNA replication can be redundant or indispensable, depending on the host cell type (4,33,34,47). Mutation in the B element does not totally abolish viral DNA replication, suggesting that enhancer A element plays the major part in viral DNA replication in NIH-3T3 cells. Furthermore, our results suggest that the B enhancer element may also facilitate Py DNA replication in NIH-3T3 cells.

Comparison of DNA synthesis of wild type and middle T antigen deficient strains. To examine the effect of middle T antigen on viral DNA synthesis in NIH-3T3 cells, we compared the replication of middle T deficient mutants, 18-5 and B2, to that of wild types, A2 and A2(eB2). Low molecular weight DNA was extracted from infected cells and analyzed by restriction endonuclease *Msp*I as described in Materials and Methods. We observed that for those pairs of viral strains with the same enhancer structure, middle T

deficient mutant replicated less efficiently than wild type in NIH-3T3 cells (Compare lanes 1 to 2, and 3 to 4, in Fig.4). Underreplication of hr-t mutant DNAs suggests that middle T and/or small T antigens stimulate viral DNA synthesis. We also compared the effect of duplicated A enhancer elements on viral DNA synthesis. Duplication of the A enhancer resulted in higher yield of DNA synthesis (Compare lanes 1 to 3, and 2 to 4, in Fig.4). Therefore, duplication in the A enhancer element exerts a *cis*-advantage for viral growth. At the level of viral DNA synthesis, the enhancer effect is of the same order of magnitude as the middle T antigen effect.

Comparing the effect of the middle T antigen and TPA on viral DNA synthesis. As discussed in the introduction, middle T antigen and TPA appear to affect cells through the same pathway. Thus it was of interest to compare their effect on viral DNA synthesis. For this purpose, infected cells were incubated in medium containing 1% serum either treated with 10 or 100 nM TPA, or mock-treated with ethanol. Under these conditions, TPA treatment had little effect on the cell division, but cell morphology was altered in that the cells appeared transformed (data not shown). The results show that TPA strongly induced DNA synthesis (approximately five folds increase) of middle T antigen deficient strains 18-5 and B2 (Fig.5). The effect was more

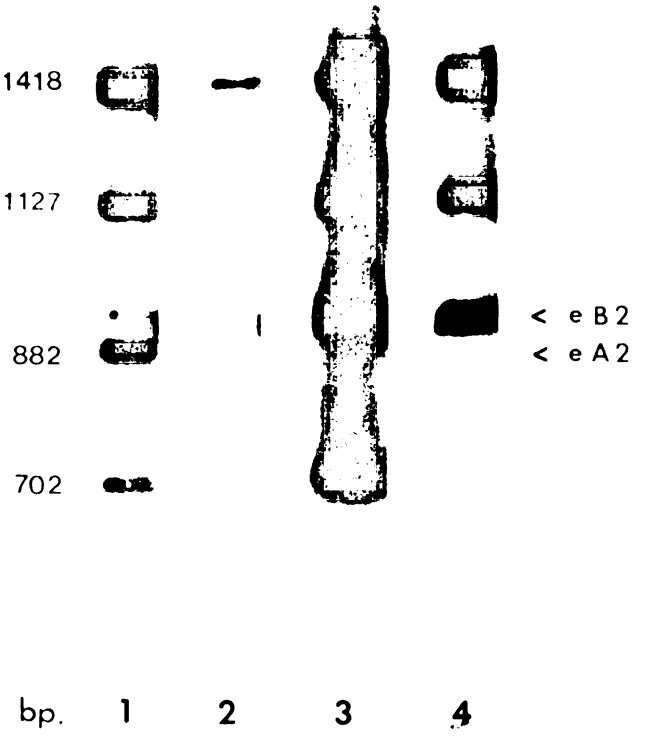


Figure 4. Effect of middle T antigen on viral DNA synthesis.

NIH-3T3 cells were infected with a middle T antigen proficient strain: A2 (lane 1), A2(eB2) (lane 3), or with a middle T deficient strain: 18-5 (lane 2), B2 (lane 4) at a multiplicity of 10 PFU/cell for each virus. Viral DNA was analyzed as described in Fig.1, and hybridized to a probe specific to the whole polyoma genome. Middle T antigen effect on viral DNA replication was compared by *Msp*I fragments #1 between viruses with a single copy of (lanes 1 and 2) and duplicated (lanes 3 and 4) A enhancer element.

pronounced for the strain 18-5, which contains a single A enhancer element (see Materials and Methods). The TPA effect did not alter the effect showing the duplication of the A enhancer, since more viral DNA replication was observed with strain B2 than with 18-5 in the presence of TPA.

In contrast, in the presence of middle T antigen, no TPA effect could be observed. Since the full effects of TPA were observed at the concentration of 10 nM for mutants, and since increasing the concentration of TPA to 100 nM in wild type infected cells had no effect on viral DNA synthesis, it appears unlikely that the absence of stimulating wild-type DNA synthesis was due to insufficient TPA concentration. Clearly, the middle T antigen is a more potent inducer of the viral DNA synthesis than TPA.

The effect of middle T antigen, TPA, and serum concentration on competition between enhancers. To examine whether TPA and middle T antigen activate Py DNA synthesis through the same target, we compared the effect of TPA, middle T antigen, and serum on the competition between viruses with single and duplicated A enhancer elements. NIH-3T3 cells were infected at equal multiplicities with pairs of strains which differ in the enhancer. One pair of viruses were middle T antigen proficient, whereas the other pair were middle T antigen deficient. Infected cells were

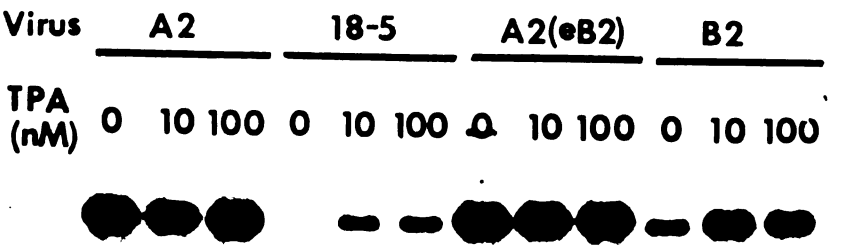


Figure 5. TPA responsiveness of viral DNA replication.

NIH-3T3 cells were infected with each virus at the multiplicity of 2 and incubated with or without TPA. Viral DNA was digested with *EcoR*I, electrophoresed on a .7% agarose gel. Hybridization was conducted with a probe for the whole viral genome (pPy-1).

incubated in medium containing either 0.5 or 5% serum, and with or without TPA (20 nM).

The activation of the cell's capacity for viral DNA synthesis by middle T antigen and TPA which was already described in Figs. 4 and 5, is demonstrated again. The stimulatory effect of TPA is particularly visible in low serum (Compare lanes 1 and 2, 5 and 6, in Fig.6). In addition, serum also increases the capacity for viral DNA synthesis (Compare lanes 1 and 3, 5 and 7, in Fig.6). Comparing the difference between lanes 1 and 2 with that between lanes 1 and 3 in Fig.6, it appears that the effect of serum is similar in magnitude to the effect of TPA and little synergy is observed with these two agents. Neither agent is as potent as middle T antigen (Compare A and B, Fig.6). Interestingly, the capacity for viral DNA synthesis is not fully utilized at the low multiplicity, since a big increase is observed by raising the multiplicity. The multiplicity effect is maximal in cells infected in low serum and without TPA treatment (Compare lanes 1 and 5, Fig.6). Only a small fraction (approximately 15%) of this effect can be attributed to an increase in the number of infected cells at the multiplicities used. The mitogenic effect of capsid attachment at the early step of infection (54) may contribute to increasing the capacity for DNA synthesis, or the number of templates may be rate limiting

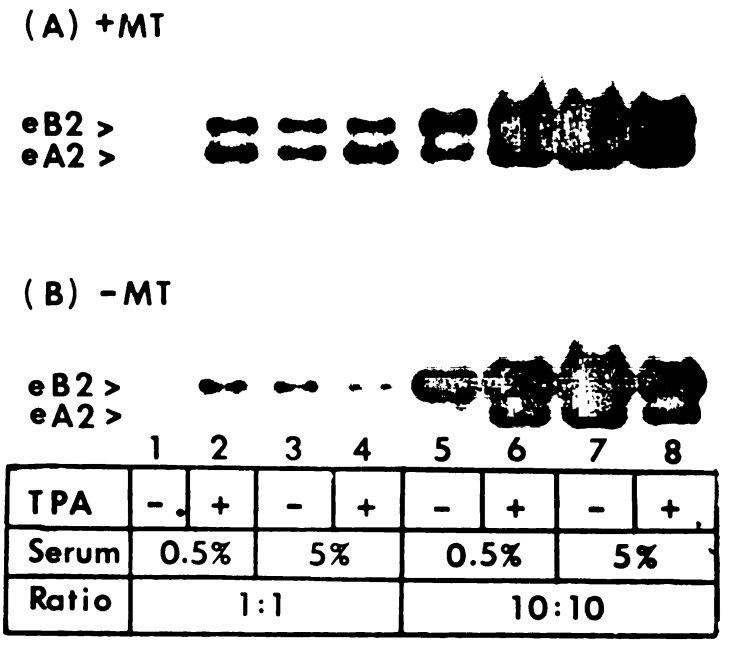


Figure 6. Effect of TPA, serum or both on competition assays.

NIH-3T3 cells were infected with a mix of middle T antigen proficient viruses (A), or middle T antigen deficient viral strains (B). The MOI ratios and incubation conditions are indicated in the bottom panel. The filters containing fractionated MspI restriction fragments were hybridized with a probe specific for the MspI fragment 3. Panel B was exposed 3 times longer than panel A. MT, middle T antigen.

at lower multiplicities. This suggests that factors other than those which bind in the A enhancer are at play.

When the fate of the two parental viruses is compared, the advantage of the template with a reiteration of the A enhancer element is obvious. In these experiments, the amount of T antigen available for replication is identical for both viral strains. The strain containing a duplicated A enhancer outcompetes a template without reiteration under most conditions: low serum, absence of TPA or of middle T antigen. The fact that all three agents: middle T antigen, TPA, and serum relieve the competition support the hypothesis that all three affect a factor required for DNA replication whose target of action lies in the A enhancer element. In the case of relieving competition, the middle T antigen appears to be the most potent agent.

The effect of duplicated A enhancer and middle T antigen in baby mouse kidney cells. Baby mouse kidney (BMK) cells have been shown to be permissive for the replication of hr-t mutants, suggesting that the activity of the middle T antigen is not essential in this cell type (15). In BMK cells, the contribution of the middle T antigen to the yield of viral DNA could not be demonstrated: middle T deficient mutants accumulated viral DNA to a similar extent as middle T proficient strains (Compare lanes 1 and 2, 3 and 4, in Fig.7). In contrast, the effect of the A enhancer duplication could be detected both in the presence and in

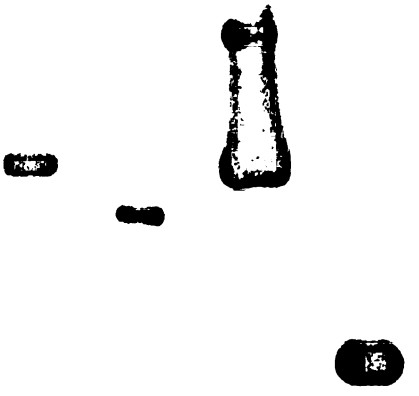


Figure 7. Viral DNA synthesis in BMK cells.

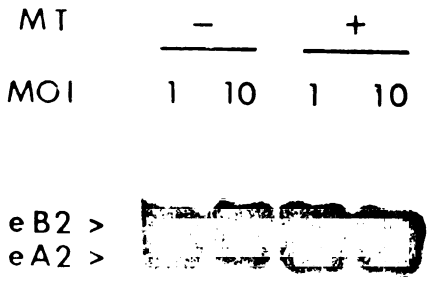
BMK cells were infected with A2 (lane 1), 18-5 (lane 2), A2(eB2) (lane 3), B2 (lane 4) at a multiplicity of 10 PFU. Low molecular weight DNA was analyzed as described in the legend to Fig.1.

the absence of the middle T antigen (Compare lanes 1 and 3, as well as lanes 2 and 4, in Fig.7). As described above for NIH-3T3 cells, the competition in viral DNA synthesis between mutant strains 18-5 and B2 were observed in BMK cells (panel A, Fig.8). Thus, the permissive factors in the BMK cells are unlike middle T antigen, which alleviates the competition between viral genomes with different A enhancers in NIH-3T3 cells. As will be discussed in detail below, the likely candidates for enhancer duplication effect in NIH-3T3 cells are PEA1 and PEA3. These factors are known to be activated by middle T antigen resulting in increased viral transcription (49). As the A enhancer duplication extends beyond PEA1 and PEA3 binding sites, it is possible that other factors are involved in BMK cells.

Moreover, we observed that in the BMK cells, strains with B2 enhancer outgrow strains with A2 enhancer (panel B, Fig.8). Therefore, in this typical cell type, the duplication of the A enhancer element exerts a strong *cis*-acting advantage for viral DNA synthesis regardless the presence of middle T antigen.

Discussion

In this study, we have shown that alterations of the enhancer have coevolved with deletions of the middle/small T antigen in natural isolates of polyoma mutants selected for



(A) (B)

Figure 8. Mixed infections of BMK cells.

BMK cells were doubly infected with either transformation-competent (+MT) or -defective (-MT) viral strains. The viral DNA was analyzed as described in Fig.6.

the growth dependence on transformation (hr-t mutants). These rearrangements of the enhancer confer a strong *cis*-advantage in viral DNA replication to any genome which bears such a rearrangement. However, the *cis*-advantage is particularly noticeable for middle T antigen deficient mutants. In fact, as shown in Fig.6, a middle T antigen deficient mutant without rearrangement is not capable of DNA replication in NIH-3T3 cells in low serum. These results provide an adequate explanation for our repeated failure to obtain such mutants by construction (Chen, Friderici and Fluck, unpublished data). The *cis*-advantage is also observable in the presence of middle T antigen.

When strains carrying rearrangements are used in mixed infections with strains carrying the A2 wild type enhancer, a strong competition is observed in which the genome with the enhancer duplication outreplicates the other genome. This effect again is much stronger in the absence of middle T antigen (i.e. in infections with 2 middle T antigen deficient mutants) or at low middle T antigen levels (i.e. in infections with a high ratio of middle T antigen deficient to middle T antigen competent genomes). This effect had been noted previously and referred to as a dominant lethal effect (12). The enhancer competition is also easily observable in rat cells in which hr-t mutants prevent wild-type from transforming (32, Chapter 4). Cell

specific *cis*-acting advantages of A enhancer duplications have been reported previously for polyoma mutants isolated from Friend erythroleukemic cells and neuroblastoma cells (9,10).

Our experiments demonstrate clearly that the target for the "dominant lethal effect" of hr-t mutants is the rearrangement of the enhancer. Mutants with multiple reiterations of the A domain compete more strongly than those with simple structure. The simplest mutant such as B2, which has been studied in detail by competition assays, shows that the target for these is located in the A domain between nucleotides 5097 and 5132. This domain is known to bind the transcription factors PEA1, PEA2, and PEA3 (27). PEA1 has been identified as the mouse homolog of AP1 (27) and PEA3 as the homolog of c-ets (48). TPA has been shown to activate transcription from the PEA1 as well as the PEA3 sites (22,53). Synergy is observed between the two sites (35,48,50). The same results have been obtained with middle T antigen (49). TPA stimulates DNA replication of a plasmid containing the PEA1 and PEA3 binding sites (30). The activation by middle T antigen of genes which are the target of protein kinase C activation is understood to be a result of the activation of the phosphatidylinositol signal transduction pathway by middle T antigen (44).

Our overall model represents a tie, which brought many separate observations that have been derived by using plasmids into the life cycle of the virus (30,51). The major new aspect is the important role for middle T antigen in DNA synthesis via the activation of enhancer binding cellular factors and thus, an important role for PEA1/PEA3 in DNA replication in the polyoma system. The experiments presented here as well as a detailed study presented in Chapter 2, suggest that factors for DNA replication are present in limiting amounts in NIH-3T3 cells. Key factors are those which bind in the A element i.e., at least PEA1/AP-1 and PEA3/c-ets. These factors can be induced by serum, TPA and middle T antigen (22,49,53), presumably via the induction of protein kinase C. Our hypothesis is that reiteration of the A element represent a way to increase the probability of binding to these limiting factors. Thus middle T antigen deficient mutants with reiterations of the A enhancer have a strong advantage over those without reiterations, in competing for the cellular factors. In the presence of the viral inducer, middle T antigen, the concentration of these factors must rise sufficiently to relieve most of the advantage associated with the duplications. Both serum and TPA can in part complement for the absence of middle T antigen, however, middle T antigen is a much more potent inducer than either of these agents.

As other polyomaviruses, polyoma enhancer contains motifs for binding multiple cellular factors (29,47). This may represent an adaptation for growth in multiple tissues in the natural host (Amalfitano et al., in preparation). An intriguing aspect of the enhancer is that it can be rearranged at high frequencies in various ways to generate mutants with new host range (1). Many of these rearrangements involve duplications. Both single copy and duplicated enhancers have been described in naturally occurring polyoma strains (36). The generation of duplications in the polyoma enhancer has been observed in many systems. In fact, the commonality of such rearrangements distracted from giving importance to the observation that hr-t mutants contained alterations in the fragments around the origin when these were first noted (11,18). The frequent generation of such duplication calls for an explanation. The position of the origin proximal to the duplication and the fact that the duplications are always situated on the late side of the origin suggest that they may arise when replication forks progress preferentially over the nucleosome free region (19) and stall when encountering the first nucleosome both on the early and on the late sides. Evidence that replication progresses faster on the late side is available for SV-40 (43). A partially duplicated molecule would result from this situation which might be at times resolved by a

recombination event generating the duplication.

In conclusion, our results suggest a strong functional interdependence between middle T antigen and the A enhancer element. This dependence is possibly mediated by the induction of the PEA1/AP1 and the PEA3/c-ets factors by middle T antigen, and by the requirement for at least these two factors for DNA replication in NIH-3T3 cells.

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Chapter 4.

The Role of the Polyomavirus Enhancer in Neoplastic Transformation[1]

ABSTRACT

We analyzed the transformation frequencies induced by polyomavirus from experiments in which FR-3T3 cells were infected with a mixed viral population containing a transforming wild-type plus a transformation-deficient mutant. The results demonstrate a dosage- and enhancer-dependent "interference effect", in that increasing the number of mutant genomes and/or reiterations of the A enhancer element in the mutant genome induced a progressive inhibition of transformation induced by wild type. Inhibition of abortive transformation and viral DNA replication of wild-type were also observed. All three effects appear to be mediated by the limiting amounts of factors which bind in the A enhancer element. One possible interpretation for the inhibition of stable transformation is that the binding of factors to the enhancer is an important step in the integration of the viral genome.

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During infection of nonpermissive cells by polyomavirus (Py), stable neoplastic transformation relies on integration of the viral genome into the host chromosome with concomitant expression of the viral oncogene, middle T antigen. This is deduced primarily from the studies of different classes of Py mutants. Infection of rat or hamster cells with host range transformation-deficient (hr-t) mutants does not cause either abortive or stable transformation of the hosts (14). The hr-t gene encodes middle T and small T antigens as dual products (27). A mutant that expresses only large T and small T antigens is unable to transform cells (22), thus middle T antigen is the transforming protein of Py. Further evidence supports the above conclusion; for instance, expression of middle T antigen from a cDNA in normal established rat fibroblasts induces full transformation (32). In contrast, large T antigen temperature sensitive mutants (ts-a) induce a normal frequency of abortive transformation but a decreased frequency of stable transformation at nonpermissive temperature (17,29). However, cells can be stably transformed by these mutants at low temperature, and further temperature shifts do not abolish the transformed phenotype (29). Moreover, cells stably transformed by Py often fail to express an intact large T antigen. Py large T antigen, therefore, is important for establishing but not for maintaining stable transformation. Complementation studies

using large T and middle T antigen-mutants demonstrate that two early proteins, large T and middle T antigens, are required for Py mediated stable transformation (for reviews, see 15). Large T antigen plays an important part in the initial steps, presumably by facilitating integration of viral genomes into host chromosome (17). Once virus integrates, continuous expression of middle T antigen is required to maintain a transformation state.

DNA analyses of cell lines transformed by wild-type Py demonstrate that integrated viral genomes are usually arranged in a head-to-tail tandem manner (2,3). In those rare tsa-transformants obtained at nonpermissive temperature less than a complete copy of the viral genome is usually integrated (12). Moreover, viral replication origin is required for Py transformation and for formation of tandem insertions (10). This implies that viral DNA synthesis plays a role in the process of integration. A previous report from our laboratory supports the role of large T antigen in integration, but argues against the existence of close correlation between Py DNA synthesis and viral induced neoplastic transformation. It suggests a function of large T antigen other than amplifying the viral genome to enhance the probability of integration. Multiple functions have been associated with large T antigen: helicase activity (28), DNA-unwinding activity (11), and recombinogenic

activity (30); however, the mechanism by which large T antigen mediates viral integration is still unclear.

We have followed an interesting observation which shows that hr-t mutants of Py inhibit the growth of wild-type and ts-a mutants in a dosage dependent manner (14). Analyses of viral DNA replication in mixed infections of permissive mouse cells demonstrate that the "dominant lethal effect" results from competition between the viral genomes for limiting cellular factors that bind specifically to the sequences in A element of the Py enhancer (Chapter 2). Interestingly, seven of the natural hr-t isolates which have been sequenced contain reiteration(s) of the A enhancer element (Chapter 3). Since middle T antigen activates Py DNA replication and gene expression through PEA1 and PEA3 motifs in the A element (34,35), we hypothesize that enhancer duplications in hr-t genomes represent a compensational event for the loss of middle T function. Results supporting the hypothesis come from experiments which show *cis*-advantage growth for an hr-t genome with a duplicated A enhancer (Chapters 2,3).

Utilizing the same assays, we tested whether hr-t mutants affect wild-type induced transformation in nonpermissive cells. We first determined whether "dominant lethal effect" exists in rat cells as well as in NIH-3T3 mouse cells. For this purpose, we carried out mixed

infections of FR-3T3 cells with three viral combinations using a wild-type strain and an hr-t deletion mutant, i.e., A2 with B2, A2 with 18-5, and A2(eB2) with B2. Other than different in the transformation capability, A2 and B2 strains contain differences in their enhancer-origin regions; whereas in the latter two pairs, both parental viruses carry the same enhancer-origin structure (21, Chapter 3). Each infection contained a fixed concentration of A2 or A2(eB2) at a multiplicity of 1. The concentrations of the 18-5 and B2 were varied by increasing the multiplicities from 1 to 20. Strains B2 and 18-5 are deletion mutants with 241 and 50 bp deletions in their middle T and small T antigen coding regions, respectively, which can be distinguished from the wild-types by the size of *Msp*I fragment #4. The relative ratios of the two parental viruses were confirmed by analyzing viral DNA in the input (data not shown). Fig.1 shows an example of the yield of viral DNA at seven days post infection. We found that DNA synthesis of A2 and A2(eB2) were decreased in the presence of B2 in a dosage dependent manner. In addition, a higher MOI of B2 was required to outcompete A2(eB2) than to outcompete A2. The dosage- and enhancer-dependent competition results in underreplication of the wild-type DNA, suggesting that FR-3T3 cells contain limiting amounts of A enhancer factors.

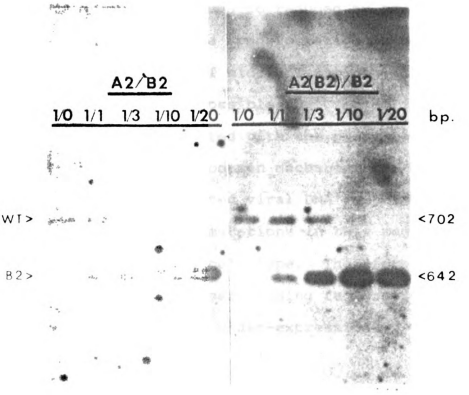


Figure 1. Viral DNA replication in mixed infection of FR-3T3 cells.

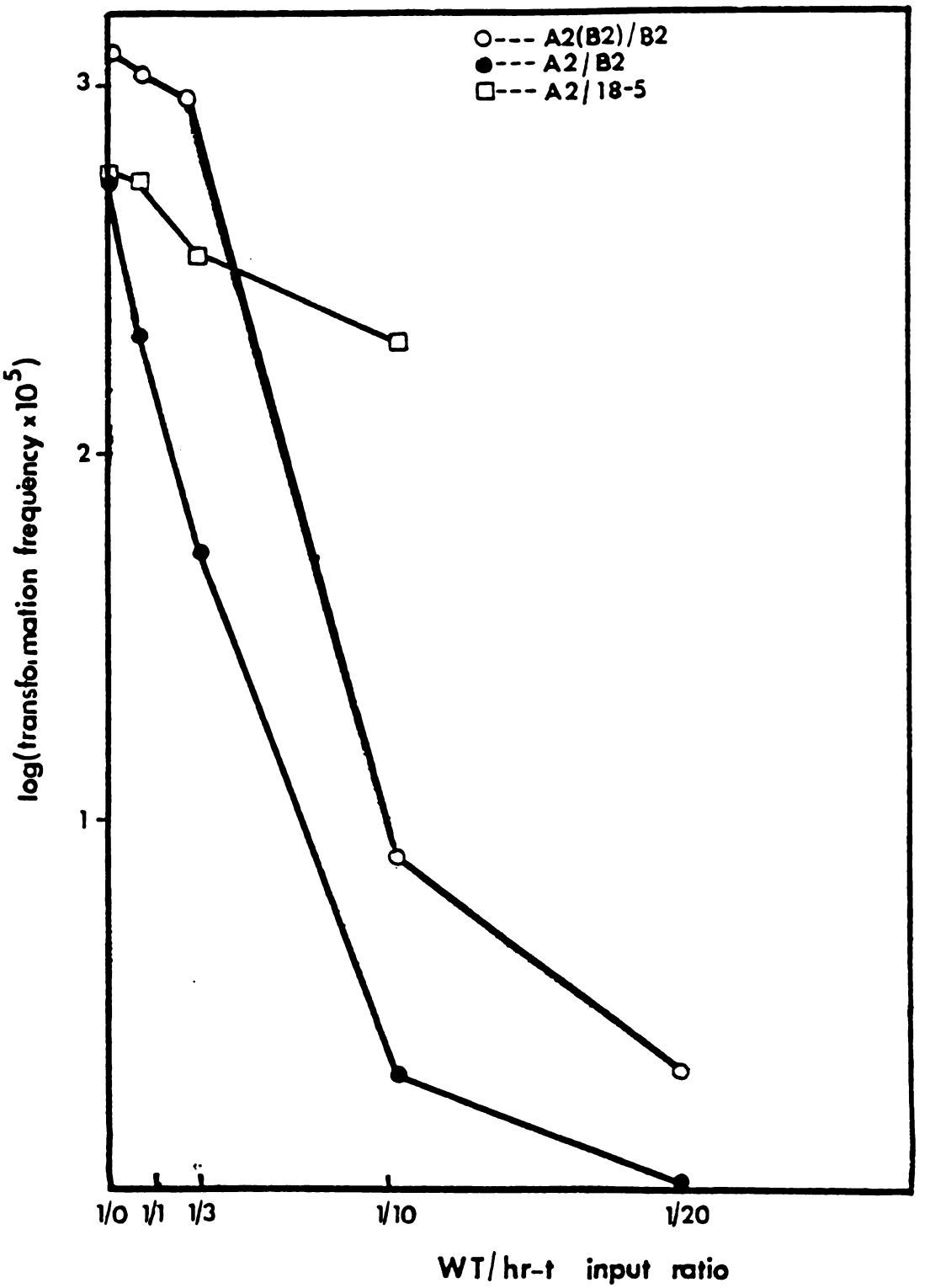
FR-3T3 cells were infected with mixtures of A2 and B2 (A), or A2(eB2) and B2 (B). The ratios of MOIs of wild-type [A2 or A2(eB2)] to mutant (B2) are given. Total DNA was collected from infected cells at seven days post infection. One tenth of each sample was analyzed by *Msp*I digestion, electrophoresed on a 2% agarose gel, transferred to nitrocellulose, and hybridized to a probe specific to *Msp*I restriction fragment #4. The upper bands represent *Msp*I-4 of A2 or A2(eB2), and the lower bands corresponds to that of B2.

During the course of infections, we found that abortive transformation frequency was also reduced by hr-t mutant. Since occurrence of abortive transformation is correlated with transient expression of middle T antigen at the early stage of infection, it is possible that transcription of the wild-type genome is interfered with the presence of excess amount of hr-t mutants. A common mechanism has been proposed for enhancer mediated viral DNA replication and transcription since single mutations in this region affect both functions in the same cell type. Therefore, competition for the A enhancer binding factors results in underreplication as well as under-expression of the wild-type genome.

Another observation we made was alterations of stable transformation frequencies in the mixed infections. Stable transformation was determined by the ability of anchorage-independent growth. Hr-t mutants are defective in transforming rat cells because of lacking functional middle T antigen, however, they integrate normally as the wild-type does (21). Therefore, transformation frequency derived from double infection represented the number of cells in the population containing integrated viral genomes expressing functional middle T antigen. The results are summarized and depicted in Fig.2. Primarily, we found that in single infections A2(eB2) induced a higher incidence of transformation than A2 did (Fig.2), suggesting that A2(eB2)

Figure 2. Transformation in mixed infections of FR-3T3 cells.

FR-3T3 cells were plated at a density of 1 x 10^5 cells per 60 mm dish. After the cells attached to the plates, they were infected with mixtures of transforming wild-type [A2 or A2(eB2)] and nontransforming hr-t mutant (B2 or 18-5) strains of polyoma. The ratios of MOIs of wild-type (WT) to hr-t are given. Transformation was scored by the appearance of colonies over the soft agar. The transformation frequency was determined by dividing the number of colonies obtained with the number of cells used in the infection.


○--- A2(B2)/B2
●--- A2/B2
□--- A2/18-5
log(transformation frequency x 10^5)
3
2
1
1/0
1/1
1/3
1/10
1/20
WT/hr-t input ratio

is a stronger transforming virus. Addition of hr-t mutant in the infection led to a decrease of transformation frequency when compared to that in the single infection. The reduction of transformation frequency was dosage-dependent, in that interference became more obvious when higher doses of hr-t mutants were applied. Transformation frequencies were less affected when parental viruses contain the same enhancer sequences, i.e., in the mixed infection of A2 and 18-5 or of A2(eB2) and B2. In contrast, A2 induced transformation was greatly inhibited by B2 when compared to the reduction of transformation frequencies in the other two pairs of infections. It suggests that the structure of enhancer element in viral genome might be involved in a rate-limiting step in the process of Py transformation.

Integration of viral DNA into one host chromosome is required for Py mediated neoplastic transformation, and has been suggested to be a rate limiting step during this process (29). To test whether reiterations of the A enhancer element affect viral DNA integrations, we carried out several mixed infections of FR-3T3 cells with two transforming viruses, A2 and A2(eX). A2(eX) represents a transformation-competent recombinant whose coding sequences are derived from A2, and enhancer-origin region is from an hr-t mutant, X (Chapter 2). Various enhancer regions of different hr-t mutants were utilized, whose sequences have been determined and shown to contain reiterations in the A

enhancer element (1, Chapter 3). A multiplicity of 10 was used for each virus in the infection. We isolated transformants from soft agar and analyzed integrated viral DNA in high molecular weight DNA. Analyses of a total of 96 transformants from eight independent infections are summarized in Table 1. Species of the integrated viral genome was determined by the size of *Msp*I fragment #3 that contained Py enhancer sequences. Fig.3 shows a representative result of DNA analysis from experiment #4 in the Table 1. Each transformant contained typical head-to-tail tandem arrays of the viral genome, since all the *Msp*I restriction fragments can be recovered. We found that only three out of 96 transformants (3%) have both parental genomes integrated. In agreement with our previous report (24), we demonstrated again that the probability for more than one integration event in Py transformation is low. In other words, it suggests that integration of a viral genome prevents a secondary integration event in the same host. In addition, half of the transformants contain A2 DNA indicating no preference for integrating a wild-type genome with duplicated A enhancer element.

In this report, we have tested the role of the Py enhancer in neoplastic transformation of nonpermissive cells. We carried out double infections of FR-3T3 cells with a fixed amount of wild-type and various amounts of hr-t

Table 1. Integration event in polyomavirus transformed FR-3T3 cell lines derived from mixed infections.[a]

	Viral strain[b]		integrations[c]		
exp. no.	WT 1	WT 2	WT 1	WT 2	Double
1.	A2	A2(eB2)	9	3	0
2.	A2	A2(eRA)	1	9	0
3.	A2	A2(eB2)	3	8	1
4.	A2	A2(eB2)	7	6	0
5a.	A2	A2(eA8)	12	1	0
5b.	A2	A2(eA8)	5	5	0
6a.	A2	A2(eA9)	7	7	2
6b.	A2	A2(eA9)	7	3	0

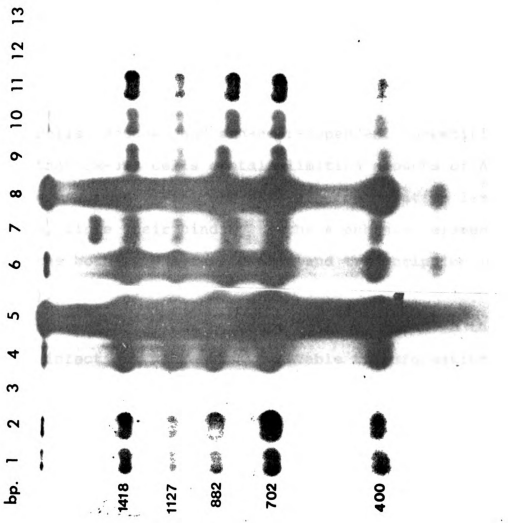
[a]Summary of the transformants analyzed in this report. FR-3T3 cells were coinfected with two transforming strains at the multiplicity ratio of 10 to 10.

[b]WT, transforming wild types used in the infections. WT1 and WT2 are different in their enhancer-origin regions which can be distinguished by *Msp*I restriction fragments #3.

[c]Summary of the number of transformants containing either one or both parental genomes analyzed as determined by Southern blotting analysis.

Figure 3. DNA analysis of transformants derived from mixed infection with two transforming strains.

FR-3T3 cells were infected with A2 and A2(eB2) at a multiplicity of infection of 10 PFU/cell of each parent (Table 1, experiment 4). A2(eB2) contains a tandem duplication in the A enhancer element, which can be distinguished from A2 by different sizes of the *Msp*I fragment #3. Transformants were cloned by anchorage independence growth in soft agar. A 10 μg portion of high-molecular-weight DNA isolated from these clones was digested with *Msp*I and was fractionated on 1.8% agarose gel. DNA was blotted to nitrocellulose paper and hybridized with a probe for the whole viral genome (pPy-1). bp, Base pairs.


bp. 1 2 3 4 5 6 7 8 9 10 11 12 13
1418
1127
882
702
400
< A2(eB2)
< A2

mutants. Two parental viruses contain the same or different sequences in the A enhancer element. Viral DNA synthesis and abortive transformation rate were analyzed during the early stage of infections. Since expression of middle T antigen early post infection induce transient anchorage independent growth, the abortive transformation rate was used as an indicator for the level of transcription from the wild-type genomes. The results showed that both DNA replication and transcription from the wild-type virus were inhibited in the presence of mutant. Similar to the NIH-3T3 mouse cells, dosage- and enhancer-dependent competitions imply that FR-3T3 cells contain limiting amounts of A enhancer factors. PEA1 and PEA3 are the putative limiting factors, since their binding to the A enhancer element activates both Py DNA replication and transcription *in cis* (34,35).

Interestingly, the presence of hr-t mutant in the double infections decreases the stable transformation frequency. We have yet to determine how hr-t mutant interferes with wild-type induced stable transformation. There are two models that we have raised: (1) the wild-type genome is prevented from integrating into the host chromosome, and (2) the expression of middle T antigen from integrated virus is suppressed. Our observation of diminished wild-type DNA replication and lower abortive

transformation rate regarding the enhancer competition favor the latter hypothesis. Above all, hr-t mutant has to exist in the same cell with wild-type to compete for viral DNA synthesis and transcription. Based on the very low efficiency of viral DNA replication in nonpermissive cells, we postulate that integration of both viral genomes into same host DNA is required for co-existence. Nevertheless, the evidence of low probability of double integration in a clonal transformant (24) argues against both viruses being in the same cell after integration occurs. Consequently, the decreased transformation frequency is unlikely to result from the suppression of middle T antigen expression from the wild-type genome by a coexisting hr-t mutant at late stage of Py infection.

It has been suggested that integration of the viral genome is a rate-limiting step in transformation on the basis of one lower frequency of stable transformants compared with that of abortive transformants (29). The elevated transformation frequency induced by A2(eB2) could be due to more viral DNA synthesis leading to a higher integration rate. The lower frequency of stable transformation in the mixed infection could result from underreplication of the wild-type genome at early stage of infection, which provides less templates for integration. The above points suggest that the Py enhancer affects neoplastic transformation by mediating the capability of

viral DNA synthesis, and the number of DNA templates is correlated with transformation frequency. However, it has been shown that the level of viral DNA synthesis does not fully account for the change of transformation frequency in Py infection of nonpermissive cells (18). In addition, in the same mixed infection we found that the decrease of stable transformation rate was more profound than those of DNA replication and transcription were, i.e., we observed decrease of stable transformation early at the input ratios of 1 to 1 and 1 to 3 (Fig.2), whereas an input ratio of 1 to 10 was needed to observe obvious competition in DNA synthesis (Fig.1). We speculate that other mechanism(s) are involved in the Py enhancer mediated viral DNA integration during neoplastic transformation.

Alternatively, we hypothesize that the Py enhancer plays a direct role as a *cis*-acting signal for an activation of the viral genome required for integration. However, analyses of integrated viral genome in transformants derived from mixed infections utilizing two transforming viruses did not prove the above postulation. Little competition between transforming wild-types has been observed in NIH-3T3 cells (Chapter 3), moreover, a strong *cis*-advantage of duplicated A enhancer element was only observed in the absence of middle T antigen (Chapters 2 and 3). Duplication of this region in the Py enhancer may represent a compensation for

the mutation of middle T antigen. Possibly cells express plenty of A enhancer factors in the presence of middle T antigen, thereby competition between viral genomes is alleviated. Since middle T antigen mediates enhancer activity and alleviates competition, it is understandable that little competition for integration occurs between transformation-competent viruses which have different copy number of A enhancer element.

Several explanations could be applied for enhancer function in integration of the Py genome. First, the existence of DNase I-hypersensitive sites in the regulatory region of viral chromosome is highly correlated with enhancer for transcription and replication (7,20). Enhancer activities of Py are dispensable for DNA replication and transcription when little chromatin structure is present, for instance, in a cell-free condition (25), and in early development of mouse embryos at the two-cell stage (23). It is generally thought that binding of nuclear proteins to enhancer sequences provides nucleosome-free areas in chromatin, which triggers signals in DNA to both replication and transcription complexes. Such a nuclease accessible region in the Py genome may enhance the probability of its recombination (integration) by altering DNA conformation. Integration of viral genome involves recombination with host DNA. Occurrence of recombination has been correlated with transcriptionally active gene (4,31), possibly due to its

DNA accessibility that is also required for initiating recombination. Second, binding of enhancer factors may facilitate interaction between viral DNA and the large T antigen, which is responsible for the typical Py integration pattern. It has been shown that subpopulation of the large T antigen are associated with the nuclear matrix (8). Enhancer may function to localize DNA at cellular structure such as the nuclear matrix. Moreover, Bourgaux et al. have indicated that proximity to the Py replication origin being a critical determining factor for recombination event (6).

Py integration occurs by nonhomologous recombination with host DNA and usually is associated with rearrangements or deletion of host DNA (3,19,36). Multiple copies of the viral genomes are commonly present in the transformed cells (2,16). Due to variable integration patterns found in different transformants and no specific viral-cellular joints yet being discovered, it is assumed that the host chromosome comprises numerous integration sites and each integration event is independent. The evidence against any specificity in SV40-transformed cells is well known (5). Similar indications of no specific integration site in host DNA have also been cited for Py (12,19,26). However, difficulties of recovering specific integration sequences might be resulted from the instability of integrated DNA in Py-transformed cells (2,9,37). We have followed the fate of Py genomes in stable transformants derived from infection of

rat cells with pairs of transforming viruses which contain differences in the enhancer region. Similar to our previous studies (24), we found very low incidence of double integration. Thus, we assume that either limited number of chromosomal sites are available for Py integration and/or limited number of viral genomes become capable to integrate. Since each viral genome has equal opportunities to integrate in these mixed infections (~50%), low rate of recovering second parental genome in a transformant suggests that the integration event is restricted.

In conclusion, competition assays demonstrated that immortalized FR-3T3 cells contain limiting amount of A enhancer factors which affect viral DNA replication, transcription, and transformation. Integration has been proposed to be a rate limiting step in stable transformation, and appears to be most affected by the presence of competitors, thus enhancer might be involved in the step of integration of Py genome into host chromosome. Furthermore, low frequency of double integrations were found in the mixed infections of transforming viruses, suggesting that integration event in a host DNA is restricted. We hypothesize that enhancer factors act as a *cis*-acting signal, by binding to the A enhancer element, which activates viral genome to integrate into the host genome.

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SUMMARY AND CONCLUSIONS

The role of polyoma enhancer region in viral DNA replication and neoplastic transformation was investigated. The experiments described in Chapter 2 used competition assays to determine that limited amount of cellular factors are present in NIH-3T3 mouse cells. Studies of enhancer variants suggest that nuclear factor(s) binding to the A enhancer element represent the major class of the candidates. The presence of this enhancer element has been shown to *cis*-activate both polyomavirus DNA replication and transcription, though the mechanism is unclear. Results from the kinetic studies suggest that function of the A enhancer element is involved in an initiation step of DNA synthesis. Possibly interactions with enhancer factors facilitate the formation of replication complex around the origin.

Numerous evidence have demonstrated that rearrangements in the polyoma enhancer affect viral growth profoundly. Combining the observations that naturally isolated host range transformation deficient (hr-t) mutants are strong competitors in viral DNA replication and that they share common sequence rearrangements in the A enhancer element, we suggest that duplicated A enhancer play a positive role in the growth of this group of mutants. The previously reported "dominant lethal effect" of hr-t mutants in mixed

infections with wild-type can thus be explained as an enhancer mediated competition effect. A enhancer duplications have been discovered in several transforming polyoma strains, which do not display strong *cis*-advantage growth. By contrast, in the absence of the reiteration, nontransforming mutants have a strong defect in viral DNA synthesis. Experiments described in Chapter 3 demonstrated that strong *cis*-advantage of the duplication carrying strain can be relieved partially by TPA or serum, and totally by middle T antigen. Binding motifs in the A enhancer for PEA1 and PEA3, the mouse homologs of AP1 and c-ets, are the most likely targets since they both respond to the activation of the above three factors. Middle T antigen is postulated to be most effective than in inducing viral DNA synthesis via the activation of PEA1 and PEA3. Thus, the reiteration of the A enhancer element has possibly evolved during the natural selection for transformation dependent growth.

The experiments in Chapter 4 studied the fate of polyoma genome in the mixed infections of nonpermissive rat fibroblast cell line, FR-3T3. Results from competition assays demonstrated that FR-3T3 cells contain limited number of A enhancer factors. These proteins appear to mediate viral DNA replication, gene expression (as indicated by abortive transformation), and stable transformation. The interference effect of hr-t mutants on wild-type induced

transformation frequencies was more pronounced than on the viral DNA replication and transcription. Taken with results generated from our and other laboratories: the number of viral genome is not highly correlated with the transformation frequency, the rarity of double integration in the polyoma transformant, and integration is a putative rate-limiting step during the course of neoplastic transformation, we propose that enhancer plays a *cis*-acting role in the integration of the viral genome.

The experiments using two transforming strains with different copy number of the A enhancer elements pursued the above hypothesis. The results showed few double integration events, whereas no preference was shown to integrate the viral genome with reiterations in the enhancer. The failure to detect the differences can be explained as follows. Middle T antigen mediates A enhancer activity by inducing sufficient cellular factors PEA1 and PEA3, and alleviates the competition between the viral genomes, thus either parental genome has a similar opportunity to interact with A enhancer factors. A system other than using middle T-mediated transformation as a selection marker would be helpful to test the hypothesis. The intriguing restriction in integration of the viral genome to only one site in the genome of individual transformants remains to be understood. There can be one specific site common in every transformant

or multiple integration sites in the host DNA, and integration of one viral genome prevents the occurrence of a secondary integration event.

Several lines have implicated that specific binding of host factor with the enhancer sequences alters chromatin structure and provides nucleosome-free regions, which may trigger the initiation steps of DNA replication and transcription. Moreover, actively transcribed genes are correlated with the occurrence of recombination. The results presented here follow the above models, therefore, study of interaction between the polyoma enhancer and host machineries may be helpful in elucidating the mechanism by which enhancer mediates DNA activity.