24941134

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 00629 2571

LIBRARY
Michigan State
University

This is to certify that the

dissertation entitled

THE ROLE OF THE POLYOMA ENHANCER REGION IN REPLICATION OF THE VIRUS IN-VITRO AND IN-VIVO

presented by

Andrea Amalfitano

has been accepted towards fulfillment
of the requirements for

PhD degree in Microbiology

Michele O. Flemington
Major professor

Date 6/14/89

MSU is an Affirmative Action/Equal Opportunity Institution

O-12771

PLACE IN RETURN BOX to remove this checkout from your record.
TO AVOID FINES return on or before date due.

DATE DUE	DATE DUE	DATE DUE
FEB 0 4 2008		

MSU Is An Affirmative Action/Equal Opportunity Institution

THE ROLE OF THE POLYOMA ENHANCER REGION IN REPLICATION OF THE VIRUS IN-VITRO AND IN-VIVO

By

Andrea Amalfitano

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

1989

6001221

ABSTRACT

THE ROLE OF THE POLYOMA ENHANCER REGION IN REPLICATION OF THE VIRUS IN-VITRO AND IN-VIVO

By

Andrea Amalfitano

The enhancer region of the mouse polyomavirus has been extensively studied in tissue culture systems. The enhancer region is required not only for early gene expression, but also for replication of the viral genome. I have studied the role of the enhancer region in directing replication of the virus in tissue culture systems and in infections of the mouse. Variant viruses were constructed which only differed in their rearranged enhancer regions. Two of these variants, A2(A8) and A2(NG-23), had rearrangements which included loss of DNA sequences between nucleotides 5150-5180 (the omega domain) of the viral genome, a region which has never been reported to be deleted in viable viruses. These two viruses were shown to replicate to wild type levels in mouse NIH-3T3 cells, demonstrating that loss of these sequences is not absolutely required for viral replication. The enhancer rearrangements of these two viruses are similar to those found in polyoma host range mutants capable of growing in

the mouse PCC4 embryonal carcinoma cell line. It was found that these viruses cannot replicate to high levels in PCC4 cells, suggesting that the omega domain is required for high levels of viral replication in this cell line. The ability of the variant viruses to replicate and persist in mouse infections was also investigated utilizing whole body in-situ hybridization and tissue DNA isolation techniques. These experiments revealed that the nucleotides 5141-5264 of the viral genome are required for high levels of viral replication in the early stages of infection of mice. The enhancer was also found to play a role in the ability of the virus to persist in mouse tissues later in infection. Overall, these results demonstrate that the polyoma enhancer region plays a pivotal role in replication of the virus in infections of the mouse, as well as in tissue culture systems.

To my wife, Susan, and our families, for without their support, my goals would be out of reach

ACKNOWLEDGEMENTS

I would like to thank Dr. Michele Fluck, not only for her guidance and support , but also for her friendship and caring throughout my graduate education.

I thank the members of my guidance committee, Drs. Jerry Dodgson, Leland Velicer, Justin McCormick, Susan Conrad, Karen Friderici, and Sandra Haslam, for their advice, and interest in my project.

I thank all of the members of the Fluck lab, specifically Sue K (and family), David, Karen, Ming-Chu, Hong-Hwa, Larry, Amy, Julie, Li Jyun, and Jyothie, because they are great people to work with.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Literature review

Introduction

The mouse polyoma virus (Py) is the prototype of the polyoma family of viruses. It is a double stranded DNA virus whose genome consists of 5297 bp utilizing the numbering system of Salzman,[1] (which will be utilized throughout this study), organized as seen in Figure 1. The genome is divided into an early region and a late region, separated by approximately 350 base pairs of a non-coding regulatory region. The early region codes for one mRNA precursor, which is differentially spliced to generate three transcripts coding for the proteins known as small, middle, and large T antigens. These proteins are essential for establishment of the lytic cycle as well as for the transformation properties of the virus. The late region also codes for one mRNA precursor, which is differentially spliced to generate the three transcripts coding for the viral capsid proteins.[77]

This literature review will focus on the non-coding region of Py, the effects this region has on viral host range and tropism in tissue culture systems, and its role in regulating infections of polyoma's natural host, the mouse.

Fig. 1 Physical map of the polyoma virus genome.

Fig. 1 Physical map of the polyoma virus genome.

The inner circle represents the location of the eight fragments released when the polyoma genome is cut with the restriction enzyme HpaII. The early and late region precursor RNAs are spliced as indicated (jagged lines represent introns) to generate the mRNAs for the T-antigens or the capsid proteins, respectively. The non-coding region flanks the origin of replication. The figure is taken from Soeda et al.[77]

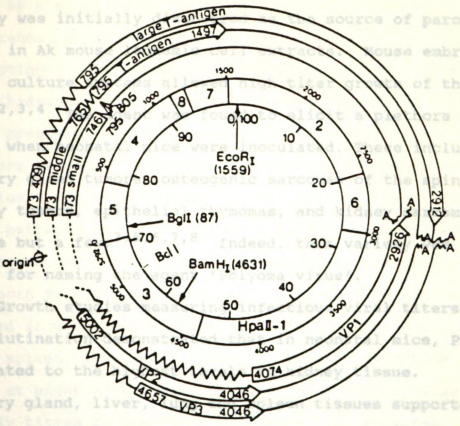


Fig. 1 Physical map of the polyoma virus genome.

Infectivity of Py in mouse tissues.

Py was initially discovered as the source of parotid tumors in Ak mouse leukemic cell extracts. Mouse embryo tissue culture systems allowed high titer growth of the agent.[2,3,4] The agent was found to elicit a plethora of tumors when neonatal mice were inoculated. These include salivary gland tumors, osteogenic sarcomas of the spine, mammary tumors, epithelial thymomas, and kidney sarcomas, to name but a few.[3,5,6,7,8] Indeed, this variety was the reason for naming the agent 'Polyoma virus'.

Growth studies measuring infectious viral titers and hemagglutination demonstrated that in neonatal mice, Py replicated to the highest levels in kidney tissue. Salivary gland, liver, lung and spleen tissues supported intermediate levels of virus replication, with brain, blood, and thymic tissues having the least ability to generate virus.[9] The highest levels of virus production is always in neonatally infected mice, which also have the highest excretion rates of infectious virus in urine, suggesting that inhalation of infectious urine may be the mechanism by which Py spreads itself through a colony of mice.[9] Transplacental transmission of virus from mother to offspring has also been demonstrated to be a possible route of viral propagation through a colony.[10]

Py is known to be present in kidney tissue of inoculated neonate mice for long periods of time, well

beyond clearance by the immune system, implicating the kidney as a major target site for viral replication, persistence, and source of infectious virus.[8,11,12] Interestingly, increased levels of virus could be detected in the kidneys of persistently infected females once they became pregnant, specifically during late gestation.[13]

More recently, utilizing in-situ hybridization and DNA isolation techniques similiar to the ones utilized in this study, patterns of Py infection in neonatally infected mice were more clearly elucidated. Intra-peritoneal infection of newborn Balb/c mice with the Py wild type A2 strain resulted in a systemic infection including the lungs, liver, spleen, and kidney tissues. Virus replication peaked at eight days post-infection, then decreased as antibody titres to the virus increased. As expected, kidney tissue could still be demonstrated to have detectable Py DNA persisting well beyond the initial immune response of the mouse.[14,15] It was noted that if neonatal mice were inoculated intra-nasally with the Py A2 strain , the same initial infection pattern was observed, however now the virus could also be demonstrated to also persist in lung tissue, a previously unknown site of Py persistence.[15]

It has been demonstrated that Py virulence in renal tissue can be changed due to strain differences, which have yet to be fully defined, or by using deletion mutants of

the early coding regions, which result in decreased ability of viruses to grow in kidney tissue.[12,16]

This study is the first to demonstrate that the Py non-coding region, (specifically the transcriptional and replicational enhancer described in the following sections), has a major role in affecting the replication of Py in infections of the mouse.

Functional mapping of the Py enhancer region

Over the years Py has proven to be a convenient system to study DNA replication and transcription in mammalian cells. It was shown that large T antigen was required in trans to initiate rounds of replication, by interacting with a short region of dyad symmetry at the origin of replication.[17] However, studies demonstrated that deletion mutants which span the PvuII restriction enzyme site at nucleotide #5130 of the Py genome resulted in decreased early mRNA expression, even though the early mRNA cap site is greater than 300 base pairs away[18] (see Figure 1). Coincidentally, deletion of this region also eliminated the ability of Py to replicate, even when large-T antigen is supplied in trans.[18] The 244 base pair BclI-PvuII restriction fragment of Py DNA (nt 5021-5262) which encompasses the PvuII site at nucleotide number 5130, was later found to contain a classical enhancer region.[20] This enhancer region was able to increase transcription of a

promoter up to three kilobase pairs away, in an orientation independent manner.[19] It was further demonstrated that Py replication requires an enhancer function per se, and that this is not specific to the enhancer utilized.[21] For example, if other known enhancer sequences (from SV-40 or the immunoglobulin enhancer) replace the Py enhancer region in plasmid constructs, replication of these plasmids can be restored. The authors suggested using Py replication as an "enhancer trap", to implicate random DNA sequences which may have transcriptional enhancer activity.[20] These studies indicated that the Py transcriptional enhancer was also required for replication, and in order to study one function or the other, these functions had to be separated, otherwise blocks in transcription would always result in blocks to replication.

To study the transcriptional activity of the Py enhancer region it was linked upstream of a beta globin promoter driving transcription of the bacterial CAT (chloramphenicol acetyltransferase) gene. These studies demonstrated that the 244 base pair enhancer region could be separated into two distinct transcriptional 'enhancer elements', called A (nt 5021-5131) and B (nt 5132-5265), each of which had different levels of enhancement in different cell lines.[21] Finer deletional analysis revealed that these two elements were actually composed of three separate transcriptional enhancer elements, any two of

which in combination can restore 100% transcriptional activity of reporter genes in mouse 3T3 cells. These three elements were called 1,2, and 3, and were mapped to nucleotides 5039-5073, 5073-5130, and 5131-5229, respectively (see Figure 2).[22]

Sequences responsible for enhancement of replication were also scrutinized. Initially, the replicational enhancer was separated into an alpha core element (nt 5097-5126) and a beta core element (nt 5172-5202),[23,24] which are functionally redundant, and in concert with the ori core in cis and large T antigen supplied in trans, allow replication of the viral genome. These elements could not enhance replication when either was placed 500 base pairs from the ori core (unlike transcriptional enhancement, which can be detected when the elements are placed up to 3 kb away from the early region promoter) suggesting that for Py both transcription and replication may require the same factors initially, but then the pathways diverge such that replication is sensitive to distances between the ori core and the replicational enhancer elements. Further studies utilizing deletional analysis lead to a finer breakdown of the replicational enhancer into four domains, (see Figure 2) each contributing to replicational enhancement, and these domains are called A,B,C, and D (located at nt 5108-5130, 5179-5214, 5148-5179, and 5020-5098, respectively).[25]

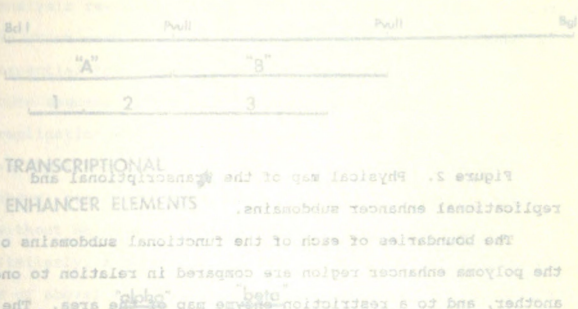


Figure 2. Physical map of the transcriptional and replicational enhancer subdomains.

Figure 2. Physical map of the transcriptional and replicational enhancer subdomains.

The boundaries of each of the functional subdomains of the polyoma enhancer region are compared in relation to one another, and to a restriction enzyme map of the area. The top figure compares the transcriptional enhancer domains, the bottom figure compares the replicational enhancer domains, utilizing the boundaries defined in the text.

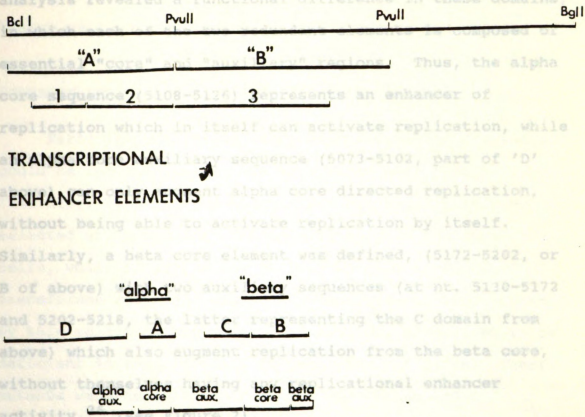


Figure 2. Physical map of the transcriptional and replicational enhancer subdomains.

Use of linker-scanning mutants in addition to deletional analysis revealed a functional difference in these domains, in which each of the two redundant elements is composed of essential "core" and "auxillary" regions. Thus, the alpha core sequence (5108-5126) represents an enhancer of replication which in itself can activate replication, while an alpha core auxiliary sequence (5073-5102, part of 'D' above) can only augment alpha core directed replication, without being able to activate replication by itself. Similarly, a beta core element was defined, (5172-5202, or B of above) with two auxiliary sequences (at nt. 5130-5172 and 5202-5218, the latter representing the C domain from above) which also augment replication from the beta core, without themselves having any replicational enhancer activity,[26] (see Figure 2).

The above studies also demonstrate that Py utilizes the same DNA sequences for replicational and transcriptional enhancement. Studies utilizing point mutations scattered throughout the enhancer region also demonstrated that the replicational and transcriptional enhancer activities cannot be separated, at least in mouse 3T6 fibroblasts.[27]

It is clear that the enhancer region of Py has adapted to allow for interactions with a variety of host cellular factors required for its transcriptional and replicational activities. The recombinant viruses constructed in this

study implicate these same regions, and further refine their roles in regards to replication of the virus in tissue culture systems and in infections of the mouse.

Enhancer region function and altered host-range

Early investigations of Py demonstrated that mutants could be isolated which have an altered host range. Initially, viruses from mutagenized stocks of virus were selected which had lost the ability to grow in mouse 3T3 cells, while retaining the ability to grow in Py transformed cells.[28] Eighteen mutant viruses were isolated by this selection procedure, and all were also found to be deficient in their ability to transform rat cells. These mutants were designated hr-t, for host range and transformation defective.[28] The non-coding regions of some recombinant viruses constructed in this study are derived from the hr-t mutants. Py hr-t mutants A8 and A9 were obtained by mutagenizing stocks with the chemical ICR-191 and isolating infectious virus, hr-t mutant II-5 was selected from a wild-type lysate after passage in Py-3T3 cells, and the hr-t mutant SD-15 was selected based upon its decreased density when banded in $CsCl_2$ gradients.[29]

Marker rescue experiments with some of these mutants mapped their host range and transformation defects to the fourth largest fragment generated by digestion of the Py genome with the restriction enzyme HpaII. However,

restriction enzyme analysis of these mutants also revealed changes in other parts of the genome, namely HpaII fragments #3 and #5 (see Fig. 1) which harbor the non-coding regulatory elements of the virus.[30] Later studies revealed that similar size differences in HpaII-fragment #3 actually represent tandem sequence duplications. For example, tandem duplications have been observed in the viral strains P16, Toronto, Ts48, MV, and NG59R.[31,67] These duplications all centered around the Py enhancer region and mimick a repeat in the SV-40 enhancer, (the 72 base pair repeat region) which is required for efficient SV-40 early gene expression.[32] It was suggested that Py, and possibly papova viruses in general, accumulate these duplications and thereby facilitate interactions with various host cells and or tissue types.[65]

The observation that various strains of Py have been found with duplications of regions important in viral expression and replication became even more striking when examining mutants of Py selected to grow on normally non-permissive cells (see Figure 3). Mouse embryonal carcinoma (EC) stem cell lines normally cannot support papova virus replication unless they are first induced to differentiate.[33] Mutants of Py were isolated which could grow on the undifferentiated form of the PCC4 EC cell line, and this new host range was found to correlate with a duplication of sequences in the A enhancer element coupled

with a deletion of sequences in the B enhancer element of the mutant viruses' genomes.[34] F9-1 EC cell lines yielded other Py host range mutants, with a single change at nucleotide #5235 (or duplication of the region encompassing this single bp change) responsible for their new tissue tropism.[35,36,37,38] It was demonstrated that a block in Py RNA expression in EC cells was removed by these altered enhancer regions.[39,40,41] Interestingly, the F9 EC mutant with a single point mutation could be demonstrated to have an altered conformation of its naked viral DNA, based upon sensitivity to the enzyme DNase I.[42]

Eventually, other Py host range mutants capable of growth in normally non-permissive cell lines were isolated. These included mutants which could grow in mouse trophoblastic, neuroblastic, and Friend leukemic cell lines.[43,44,45] All of these mutants also have rearranged enhancer regions, (see Figure 3), mainly duplications coupled with deletions, responsible for their altered host ranges. Rearrangements of enhancer regions with coincidental alterations of host range have also been observed for other polyoma viruses. For example, JC virus, the human polyomavirus, exhibits rearrangements of its enhancer which are responsible for determining the tissue tropism of various strains of the virus in human infections.[46,47]

Figure 3. Physical map of the enhancer region of some wild type and host range mutant SV viruses.

Figure 3. Physical map of the enhancer regions of some natural variant and host range mutant Py viruses.

This figure is taken from Amati (ref. 45) and compares the enhancer region modifications of natural variant polyoma viruses CSP, MV, TOR, and P16, to the rearrangements seen in host range mutants capable of growing in embryonal carcinoma (PCC4-204, PCC4 97, F9-5,and F101) trophoblastic (Tr 91, Tr 92) Friend leukemic (FL78) and neuroblastoma cell lines (NB 11/1, NB 10/6).

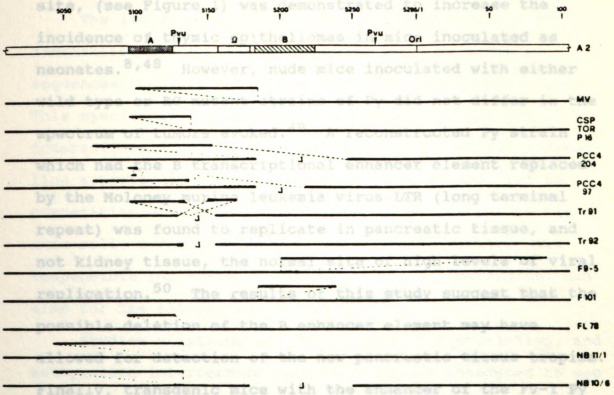


Figure 3. Physical map of the enhancer regions of some wild type and host range mutant Py viruses.

The ability of the non-coding region to influence Py replication in mouse tissues has also been investigated. Mice inoculated with a variant of Py which has a 40 base-pair duplication proximal to the Bgl I restriction enzyme site, (see Figure 1) was demonstrated to increase the incidence of thymic epitheliomas in mice inoculated as neonates.[8,48] However, nude mice inoculated with either wild type or EC mutant strains of Py did not differ in the spectrum of tumors evoked.[49] A reconstructed Py strain which had the B transcriptional enhancer element replaced by the Moloney murine leukemia virus LTR (long terminal repeat) was found to replicate in pancreatic tissue, and not kidney tissue, the normal site of high levels of viral replication.[50] The results of this study suggest that the possible deletion of the B enhancer element may have allowed for detection of the new pancreatic tissue tropism. Finally, transgenic mice with the enhancer of the F9-1 Py EC mutant driving the CAT gene did so much more effectively than when the wild-type enhancer region was used, even though both CAT gene constructs were demonstrated to be active in the same tissues.[51]

This study establishes the importance of the Py enhancer region in directing high levels of replication and persistence in mouse tissues. The recombinant viruses constructed in this study have rearrangements similar to those described for host range variants generated in tissue

culture systems, underscoring their importance in the biology of Py.

Factors which interact with the Py enhancer

The isolation of various host-range mutants of Py demonstrated that the regulatory activity of enhancer sequences themselves could account for altered host range. This specificity of activity was shown to be due to interactions with trans-acting factors of the host. Base line activity of certain enhancers could be modified by competition with other enhancer sequences present in the same cell, demonstrating that trans-acting factors were responsible for enhancer activation and or repression, and also for the altered host range.[52,53]

Studies utilizing gel shift, DNase I footprinting, and methylation interference-protection assays attempted to map the exact location of specific host-protein and viral-DNA interactions. The Py B enhancer element was the first portion of the Py enhancer to be investigated. The B enhancer element as defined by previous functional analysis[22], was shown to specifically interact with nuclear protein factors from murine cellular extracts. More precisely, nucleotides 5174-5229 of enhancer element B were implicated as the major target site for a factor subsequently called PEB-1, (for Py enhancer binding factor 1). The binding of PEB-1 absolutely required the 25 base

pairs encompassing the early proximal portion of the GC rich palindrome sequence and the SV-40 enhancer core homology contained within nucleotides 5174-5229 (see Figure 4).[54,55,56]

Other studies demonstrated that the C enhancer domain of Py, (as defined in ref. 26), was interacting with a factor in differentiated or undifferentiated F9 EC cells, as well as in mouse 3T6 cells. This factor was called EF-C, (for enhancer binding factor to Py element-C).[57,58] The EF-C binding domain is conserved in all Py viruses to date, implicating it as important in the biology of the virus. In this study, a number of recombinant viruses were sequenced and found to be lacking the EF-C binding domain, and their host range in tissue culture as well as in infections of the mouse is described. Whether or not EF-C and EBP-1 interact with each other has not been investigated. Others have described a 20,000 kD nuclear protein isolated from rat crude liver extracts which was purified based upon its strong interaction with the Py B element, (specifically, nucleotides 5211-5233, see Figure 4), and called it EBP-20, (for enhancer binding protein of 20 kD.).[59,60] Finally, it was demonstrated that a Py EC mutant, PyF441, which had a single nucleotide mutation in its B element at nt. #5235 (responsible for its ability to grow in undifferentiated F9 EC cells), could now interact with a cellular factor in undifferentiated F9 EC cells,

Figure 4. Factors which bind to the enhancer region.

Figure 4. Factors which bind to the enhancer region.

The binding sites for some of the factors described in the text are compared to the restriction enzyme map of the polyoma virus enhancer region.

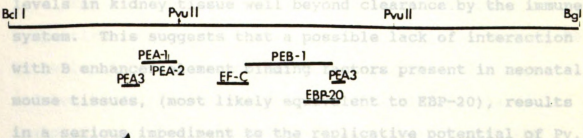


Figure 4. Factors which bind to the enhancer region.

whereas a wild-type sequence of this same region could not, suggesting a newly created positive acting sequence motif was affecting the new host range of the virus.[61] Together, these studies suggest that the functional B enhancer element of Py is the site of interaction with numerous cellular factors. In this study, the B element has been implicated as a region critical to Py not only in establishing an early infection of mice, but also in allowing the virus to be able to persist at detectable levels in kidney tissue well beyond clearance by the immune system. This suggests that a possible lack of interaction with B enhancer element binding factors present in neonatal mouse tissues, (most likely equivalent to EBP-20), results in a serious impediment to the replicative potential of Py.

The A enhancer element of Py has an even more complicated array of factors interacting with it. Utilizing a modified DNase I footprinting technique, the 22 base pair core of the alpha domain, (as defined by ref.26, nucleotides 5108-5130), was found to be the recognition site for two different proteins with overlapping binding sites. They were called PEA1 and PEA2, (for Py enhancer A binding factors 1 and 2).[62] PEA1 binds to nucleotides 5115-5125 and PEA2 to nucleotides 5122-5130 (see Figure 4). This finding, along with other studies[63], suggested that two recognition sites for trans acting proteins could evolve from a common recognition sequence.[62,63] The

sequence rearrangements of the recombinant viruses examined in this study also support this hypothesis. It was also demonstrated that PEA1 and PEA2 binding was cooperative, and that this association could induce DNase I hypersensitive sites in the viral minichromosome.[69] These DNase I hypersensitive sites were suggested to be facilitating viral DNA replication. These "core recognition sequence motifs" can be duplicated to create functional enhancer elements in SV40 as well.[64]

PEA1 and PEA2 were not found to bind to their respective recognition sites in undifferentiated PCC4 or F9 EC cells, but when these same non-permissive cells were induced to differentiate, PEA1 and PEA2 binding was restored, correlating binding of these factors with the ability of the virus to replicate in previously non-permissive cells.[65] In agreement with the above inference is the finding that Py PCC4 EC mutants accumulate duplications of their alpha domains to allow growth in undifferentiated PCC4 EC cells, which may allow the virus a better opportunity to interact with PEA1 and PEA2.[66] A third protein, PEA3, was also found to bind the Py A enhancer element at nucleotides 5108-5113, and possibly to B element sequences at nucleotides 5203-5208 (see Fig.4), and this binding allowed viral transcription and replication in mouse fibroblasts.[67]

Negatively acting factors which interact with the Py enhancer have also been investigated.[54,55,68] The clearest example of negative regulation involves the adenovirus E1a protein. When introduced into mouse cells along with a reporter gene linked either to the Py or SV40 enhancer, E1a is able to repress transcription of the reporter gene.[69] The observation that an adenovirus mutant with a defective E1a protein can grow in undifferentiated EC cells suggested that an endogenous 'E1a like' activity is present in these cells, and that this may be the reason why Py and SV40 cannot replicate in undifferentiated EC cells.[70] For example, a labile protein was implicated as preventing Py replication in undifferentiated PCC4 cells.[71] Previously discussed positively acting factors which were correlated with Py host range may in fact be modulated by these 'E1a like' negatively acting factors, and thus these observations are not mutually exclusive. Indeed, the observations underscore the fact that the Py enhancer has evolved in response to the intricate cellular control mechanisms responsible for modulating gene expression.

Other interactions with the Py enhancer region have also been noted. It was observed that the tumor promoter TPA (12-O-tetra-decanoylphorbol-13-acetate), can stimulate Py enhancer-linked expression, and that this expression was different depending on the differentiation state of the host cells.[72] The c-Ha-ras proto-oncogene in and of itself

can stimulate Py transcriptional enhancer function, and a mutated c-Ha-ras that is oncogenic can further stimulate the Py enhancer, via a trans-acting factor.[73,74] The sequence in the enhancer region responding to these various transforming oncogenes, including the middle T protein of Py, TPA, or even serum, maps to the PEA1 binding domain, indicating that the pathway of transformation is exquisitely linked to the pathway for Py enhancer activation.[75,76] Possibly Py utilizes its middle T protein to facilitate interactions with PEA1, allowing its own genome to be preferentially expressed and replicated, thus giving a "transforming protein" an actual role in the lytic cycle of the virus.

The complexity of the factor interactions within the Py enhancer region underscores the region's importance in directing transcription, replication, and the tissue specificity of the virus. The factors which have been demonstrated to bind within this region are thus implicated as being important participants in gene expression and DNA replication.

Summary

This literature review has focused upon the ability of the mouse polyoma virus to interact with a number of mouse tissues and various tissue culture cell lines, and demonstrated how the enhancer region of polyoma has been

implicated as a major determinant in these interactions. This complexity allows investigators to investigate virus-host interactions utilizing a number of various model systems. This study will describe the construction of new polyoma viruses with unique rearrangements of their enhancer regions. These reconstructed viruses allow us to ask how these rearrangements may alter the ability of the virus to grow in various tissue culture cell lines, and in tissues of the mouse, the virus's natural host.

References

1. Salzman, N.P. ed. (1986). The Papovaviridiae. Volume I: The Polyomaviruses. Plenum Press, New York and London.

2. Gross, L., (1953). A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. Proc. Soc. Exp. Biol. Med. 83: 414-421.

3. Stewart, S.E., Eddy, B.E., Borgese, N. (1958). Neoplasms in mice inoculated with a tumor agent carried in tissue culture. Journal of the National Cancer Inst. 20:1223-1243.

4. Rowe, W.P., Hartley, J.W., Estes, J.D., Huebner, R.J. (1959). Studies of mouse polyoma virus infection. I. Procedures for quantitation and detection of virus. J. Exper. Med. 109: 379-391.

5. Dawe, C.J. (1960). Cell sensitivity and specificity of response to polyoma virus. J. National Cancer Inst. Monograph No. 4. Symposium on Phenomenon of the Tumor Viruses. 67-92.

6. McCance, D.J., Sebesteny, A., Griffin, B.E., Balkwill, F., Tilly, R., Gregson, N.A. (1983). A paralytic disease in nude mice associated with polyoma virus infection. J. Gen. Virol. 64: 57-67.

7. Harper, J.S., Dawe, C.J., Trapp, B.D., McKeever, P.E., Collins, M., Woyclechowska, J.L., Madden, D.L., Sever, J.L. (1983) Paralysis in nude mice caused by polyomavirus-induced vertebral tumors, in; Polyomaviruses and Human Neurological Diseases. 359-367.

8. Dawe, C.J., Freund, R., Mandel, G., Ballmer-Hofer, K., Talmadge, D.A., Benjamin, T.L. (1987). Variations in polyoma virus genotype in relation to tumor induction in mice. Amer. J. Path. 127:243-261.

9. Rowe, W.P. (1961). The epidemiology of mouse polyoma virus infection. Bacteriol. Revs. 25:18-31.

10. McCance, D.J., Mims, C.A. (1977). Transplacental transmission of polyoma virus in mice. Infection and Immunity 18:196-202.

11. McCance, D.J. (1981). Growth and persistence of polyoma early region deletion mutants in mice. J. Virology 39:958-962.

12. Buffet, R.F., Levinthal, J.D. (1962). Polyoma virus infection in mice. Pathogenesis Arch. Pathol. (Chicago) 74: 513-526.

13. McCance, D.J., Mims, C.A. (1979). Reactivation of polyoma virus in kidneys of persistently infected mice during pregnancy. Infection and Immunity 25:998-1002.

14. Dubensky, T.W., Villarreal, L.P. (1984). The primary site of replication alters the eventual site of persistant infection by polyomavirus in mice. J. Virology 50:541-546.

15. Dubensky, T.W., Murphy, F.A., Villarreal, L.P. (1984). Detection of DNA and RNA virus genomes in organ systems of whole mice: Patterns of mouse organ infection by polyomavirus. J. Virology 50:779-783.

16. Bolen, J.B., Fisher, S.E., Chowdhury, K., Shan, T.C., Williams, J.E., Dawe, C.J., Israel, M.A. (1985). A determinant of polyomavirus virulence enhances virus growth in cells of renal origin. J. Virology 53:335-339.

17. Francke, B., Eckhart, W. (1973). Polyoma gene function required for viral DNA synthesis. Virology 55: 127-135).

18. Tyndall, C., LaMantia, G., Thacker, C.M., Favaloro, J., Kamen, R. (1981). A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nuc. Acids Res. 9:6231-6250.

19. deVilliers, J., Schaffner, W. (1981) A small segment of polyoma virus DNA enhances the expression of a cloned beta-globin gene over a distance of 1400 base pairs. Nucleic Acids Research 9:6251-6264.

20. deVilliers, J., Schaffner, W., Tyndall, C., Lupton, S., Kamen, R. (1984). Polyoma virus DNA replication requires an enhancer. Nature (London) 312:242-246.

21. Herbomel, P., Bourachot, B., Yaniv, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39:653-662.

22. Hassell, J.A., Muller, W.J., Mueller, C.R. (1984). The dual role of the polyomavirus enhancer in transcription and DNA replication. Cancer Cells 4: DNA tumor viruses, 561-569.

23. Muller, W.J., Mueller, C.R., Mes, A.M., Hassell, J.A. (1983). Polyomavirus origin for DNA replication comprises multiple genetic elements. J. Virology 47: 586-599.

24. Hassell, J.A., Muller, W.J., Mueller, C.R. (1984). The dual role of the polyomavirus enhancer in transcription and DNA replication. Cancer Cells 4: DNA tumor viruses, 561-569.

25. Veldman, G.M., Lupton, S. Kamen, R. (1985). Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. 5:649-658.

26. Muller, W.J., Dufort, D., Hassell, J. (1988). Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. Mol. Cell. Biol. 8:5000-5015.

27. Tang. W.J., Berger, S.L., Triezenberg, S.J., Folk, W.R. (1987). Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. Mol. Cell. Biol. 7:1681-1690.

28. Benjamin, T.L. (1970). Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. USA 67: 394-399.

29. Staneloni, R.J., Fluck, M.M., Benjamin, T.L. (1977). Host range selection of transformation-defective hr-t mutants of polyoma virus. Virology 77:598-609.

30. Hattori, J., Carmichael, G.G., Benjamin, T.L. (1979). DNA sequence alterations in hr-t deletion mutants of polyoma virus. Cell 16: 505-513.

31. Rothwell, V.M., Folk, W.R. (1983). Comparison of the DNA sequence of the Crawford small-plaque variant of polyomavirus with those of polyomaviruses A2 and strain 3. J. Virology 48:472-480.

32. Benoist, C., Chambon, P. (1981). In vivo sequence requirements of the SV40 early promoter region. Nature (London) 290: 304-310.

33. Fujimura, F.K., Silbert, P.E., Eckhart, W., Linney, E. (1981). Polyoma virus infection of retinoic acid-induced differentiated teratocarcinoma cells. J. Virology 39: 306-312.

34. Vasseur, M., Kress, C., Montreau, N., Blangy, D. (1980). Isolation and characterization of polyoma virus mutants able to develop in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 77: 1068-1072.

35. Katinka, M., Vasseur, M., Montreau, N., Yaniv, M., Blangy, D. (1981). Polyoma DNA sequences involved in control of viral gene expression in murine embryonal carcinoma cells. Nature (London) 290:720-722.

36. Vasseur, M., Katinka, M., Herbomel, P.J., Yaniv, M., Blangy, D. (1982). Physical and biological features of polyoma virus mutants able to infect embryonal carcinoma cell lines. J. Virology 43:800-808.

37. Katinka, M., Yaniv, M., Vasseur, M., Blangy, D. (1980). Expression of polyoma early functions in mouse embryonal carcinoma cells depends on sequence rearrangements in the beginning of the late region. Cell 20:393-399.

38. Fujimura, F.K., Deininger, P.L., Friedmann, T., Linney, E. (1981). Mutation near the polyoma DNA replication origin permits productive infection of F9 embryonal carcinoma cells. Cell 23: 809-814.

39. Dandolo, L., Blangy, D., Kamen, R. (1983) Regulation of polyoma virus transcription in murine embryonal carcinoma cells. J. Virology 47:55-64

40. Satake, M., Furukawa, K., Ito, Y. (1988). Biological activities of oligonucleotides spanning the F9 point mutation within the enhancer region of polyomavirus DNA. J. Virology 62:970-977.

41. Bohnlein, E., Chowdhury, K., Gruss, P. (1985). Functional analysis of the regulatory region of polyoma mutant F9-1 DNA. Nucl. Acids Res. 13:4789-4809.

42. Fujimura, F.K. (1988). Point mutation in the polyomavirus enhancer alters local conformation. Nuc. Acids Res. 16: 1987-1997.

43. Tanaka, K., Chowdhury, K., Chang, K.S.S., Israel, M., Ito,Y. (1982). Isolation and characterization of polyoma virus mutants which grow in murine embryonal carcinoma and trophoblast cells. EMBO 1:1521-1527.

44. Maione, R., Passananti, C., De Simone, V., Delli-Bovi, P., Augusti-Tocco, G., Amati, P. (1985). Selection of mouse neuroblastoma cell-specific polyoma virus mutants with stage differentiative advantages of replication. EMBO 4: 3215-3221.

45. DeSimone, V., Amati, P. (1987). Replicative cis-advantage of polyomavirus regulatory region mutants in different murine cell lines. J. Virology 61:1615-1620.

46. Feigenbaum, L., Khalili, K., Major, E., Khoury, G. (1987). Regulation of the host range of human papovavirus JCV. Proc. Natl. Acad. Sci. USA. 84:3695-3698.

47. Loeber, G. Dorries, K. (1988). DNA rearrangements in organ-specific variants of polyomavirus JC strain GS. J. Virology 62:1730-1735.

48. Freund, R., Dawe, C.J., Benjamin, T.L. (1988). Duplication of noncoding sequences in polyomavirus specifically augments the development of thymic tumors in mice. J. Virology 62:3896-3899.

49. Berebbi, M., Dandolo, L., Hassoun, J., Bernard, A.M., Blangy, D. (1988). Specific tissue targeting of polyoma virus oncogenicity in athymic nude mice. Oncogene 2: 149-156.

50. Rochford, R., Campbell, B.A., Villarreal, L.P. (1987). A pancreas specificity results from the combination of polyomavirus and Moloney murine leukemia virus enhancer. Proc. Natl. Acad. Sci. USA 84: 449-453.

51. Krippl, B, Griep, A.E., Mahon, K.A., Bohnlein, E., Gruss, P., Westphal, H. (1988). Expression and amplification in transgenic mice of a polyoma virus mutant regulatory region. Nuc. Acids Res. 16: 8963-8976.

52. Scholer, H.R., Gruss, P. (1984). Specific interactions between enhancer-containing molecules and cellular components. Cell 36:403-411.

53. Sleigh, M.J., Lockett, T.J., Kelly, J., Lewy, D. (1987). Competition studies with repressors and activators of viral enhancer function in F9 mouse embryonal carcinoma cells. Nuc. Acids Res. 15: 4307-4324.

54. Bohnlein, E., Gruss, P. (1986). Interaction of distinct nuclear proteins with sequences controlling the expression of polyomavirus early genes. Mol. Cell. Biol. 6:1401-1411.

55. Piette, J., Kryszke, M.H., Yaniv, M. (1985). Specific interaction of cellular factors with the B enhancer of polyoma virus. EMBO 4:2675-2685.

56. Piette, J., Yaniv, M. (1986). Molecular analysis of the interaction between an enhancer binding factor and its DNA target. Nucl. Acids Res. 14:9595-9611.

57. Fujimura, F. (1986). Nuclear activity from F9 embryonal carcinoma cells binding specifically to the enhancers of wild-type polyoma virus and Py EC mutant DNAs. Nuc. Acids Res. 14:2845-2861.

58. Ostapchuk, P., Diffley, J.F.X., Bruder, J.T., Stillman, B., Levine, A.J., Hearing, P. (1986). Interaction of a nuclear factor with the polyomavirus enhancer region. Proc. Natl. Acad. Sci. USA 83:8550-8554.

59. Graves, B.J., Johnson, P.F., McKnight, S.L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell 44:565-576.

60. Johnson, P.F., Landschulz, W.H., Graves, B.J., McKnight, S.L. (1987). Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes and Development 1:133-146.

61. Kovesdi, I., Satake, M., Furukawa, K., Reichel, R., Ito, Y., Nevins, J.R. (1987). A factor discriminating between the wild-type and a mutant polyomavirus enhancer. Nature (London) 328:87-89.

62. Piette, J., Yaniv, M. (1987). Two different factors bind to the alpha domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. EMBO 6:1331-1337.

63. Ruley, H.E., Fried, M. (1983). Sequence repeats in a polyoma virus DNA region important for gene expression. J. Virology 47:233-237.

64. Shephard, A., Clarke, J., Herr, W. (1988). Simian virus 40 revertant enhancers exhibit restricted host ranges for enhancer function.

65. Kryszke, M.H., Piette, J., Yaniv, M. (1987). Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. Nature (London) 328:254-256.

66. Melin, F., Pinon, H., Reiss, C., Kress, C., Montreau, N., Blangy, D. (1985) Common features of polyomavirus mutants selected on PCC4 embryonal carcinoma cells. EMBO 4: 1799-1803.

67. Martin, M.E., Piette, J., Yaniv, M. Tang, W.J., Folk, W.R. (1988). Activation of the polyomavirus enhancer by a murine activator protein 1 (AP1) homolog and two contiguous proteins. Proc. Natl. Acad. Sci. USA 85:5839-5843.

68. Borrelli, E., Hen, R., Chambon, P. (1984). Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature (London) 312:608-612.

69. Velcich, A., Kern, F.G., Basilico, C., Ziff, E.B. (1986). Adenovirus E1a proteins repress expression from polyomavirus early and late promoters. Mol. Cell. Biol. 6: 4019-4025.

70. Imperiale, M.J., Kao, H.T., Feldman, L., Nevins, J., Strickland, S. (1984). Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1a like activity. Mol. Cell. Biol. 4: 867-874.

71. Cremisi, C., Babinet, C. (1986). Negative regulation of early polyomavirus expression in mouse embryonal carcinoma cells. J. Virology 59:761-763.

72. Wasylyk, B., Imler, J.L., Chatton, B., Schatz, C., Wasylyk, C. (1988). Negative and positive factors determine the activity of the polyoma virus enhancer alpha domain in undifferentiated and differentiated cell types. Proc. Natl,. Acad. Sci. USA 85:7952-7956.

73. Wasylyk, C., Imler, J.L., Mutul-Perez, J. Wasylyk, B. (1987). The c-Ha-ras oncogene and a tumor promoter activate the polyoma virus enhancer. Cell 48:525-534.

74. Imler, J.L., Schatz, C., Wasylyk, C., Chatton, B., Wasylyk, B. (1988). A Harvey-ras responsive transcription element is also responsive to a tumour-promoter and to serum. Nature (London) 332: 275-278.

75. Wasylyk, C., Imler, J.l., Wasylyk, B. (1988). Transforming but not immortalizing oncogenes activate the transcription factor PEA1. EMBO 7: 2475-2483.

76. Yamaguchi, Y., Masanobu, S., Ito, Y. (1989). Two overlapping sequence motifs within the polyomavirus enhancer are independantly the targets of stimulation by both the tumor promoter 12-o-tetradecanoylphorbol-13-acetate and the Ha-ras oncogene. J. Virology 63: 1040-1048.

77. Soeda, E., Arrand, J.R., Smolar, N., Walsh, J.E., Griffin, B.E. (1980). Coding potential and regulatory signals of the polyoma virus genome. Nature (London) 283:445:453.

Chapter 2

Polyoma variants with altered enhancer regions redefine subdomains required for replication in tissue culture systems

ABSTRACT

We have constructed five new polyoma viruses which differ only in their enhancer regions, and describe the DNA sequence of their enhancer containing non-coding regions. Two of the viruses, A2(A8), and A2(NG-23), have extensive duplications coupled with a deletion of 123 bp which removes a functional subdomain of the enhancer previously suggested to be required for viral replication. These viruses are shown to replicate to wild type levels upon infection of mouse NIH-3T3 cells, suggesting that extensive duplications of enhancer subdomains can functionally replace domains thought to be indispensable. The viruses constructed resemble previously described host range mutants of polyoma which can grow in the PCC4 embryonal carcinoma cell line. Thus the mutant viruses allowed us to define which regions of the enhancer are required for growth in PCC4 cells.

INTRODUCTION

Polyoma virus has been used extensively as a probe to understand mammalian gene expression and DNA replication. In particular, the polyoma virus transcriptional and replicational enhancer region has been shown to demonstrate a functional complexity which allows for multiple interactions with various features of the host cell machinery involved in gene expression and replication.[22,26,37] Indeed, the 242 base pair BclI-PvuII fragment between nucleotides 5023 and 5265 (using the numbering system of Salzman[35]) has been exhaustively dissected into a number of subdomains, whose boundaries are compared in Figure 6. This fragment was initially described as containing two functionally redundant replicational enhancer elements, either of which positioned in cis with the ori core region allows for efficient viral replication.[26,28] This region was further subdivided into four functionally redundant subdomains named A,B,C, and D, based upon deletional analysis.[48] Later, deletional analysis coupled with linker scanning analysis divided the replicational enhancer region into two functionally redundant 'enhancer core' sequences, (which are named alpha and beta) able to activate replication by themselves (in cis with the ori core) and 'auxiliary domains' (one for the

the alpha core, two flanking the beta core) which augment core directed replication, without having any intrinsic replication enhancer activity of their own.[27]

Mapping to this same region are three functionally redundant transcriptional enhancer elements, any two of which when present restore full gene expression to linked reporter genes.[12] Polyoma is unique in that a transcriptional enhancer function per se is required for replication of the viral genome, suggesting that both transcriptional and replicational activities of the virus are sharing similar factors, and or pathways.[9,12,42,44,48] A plethora of factors have been demonstrated to interact with the polyoma enhancer region [3,17,29,30,32],(see Figure 6).

Finally, a number of mutations in polyoma viruses have been isolated consisting of rearranged enhancer regions which are responsible for altered tropism of the virus with regard to replication in various cell lines.[1,6,7,10,11,18,19,23,41,45,46] These mutations implicate viral interactions with enhancer binding host factors as one determinant of the viral host range.[20,21,29] In this study we have constructed five new viable polyoma virus strains which have altered enhancer regions, (similar to previously described PCC4 EC host range polyoma mutations). These alterations shed light on the role of subdomains of the

enhancer in replication, and in determining the host range of the virus.

Materials and Methods

Construction of recombinant viruses:

Viral DNA from five hr-t mutants was isolated from infected NIH-3T3 cells via the method of Hirt.[16] For mutants NG-23 and II-5, viral DNA was digested with the restriction enzymes BclI and BglI. The two DNA fragments were separated by electrophoresis in a low-melting temperature agarose gel, and the small Bcl-BglI fragment of each mutant was excised. For hr-t mutants A8, A9, and SD-15, purified DNA was cleaved with BamHI, ligated to BamHI cleaved pBR322 plasmid, and used to transform $dam^{-}dcm^{-}$ E. Coli using established techniques. These plasmids are referred to as pA8, pA9, and pSD-15, respectively. Plasmid DNA was isolated, cut with BclI and BglI, and the BclI-BglI small fragment of each mutant was isolated as above. For all five mutants, the small BclI-BglI fragment was ligated to the low melting temperature agarose purified BclI-BglI large fragment of Hirt isolated A2 strain DNA using the ligation conditions described by Struhl.[40]

The ligated semi-solid reaction mixtures were heated to 65^{o}C, diluted with an equivalent volume of DEAE-Dextran (1mg/ml), and used to transfect $1x10^{4}$ NIH-3T3 mouse cells on a 35mm dish. Virus production was visualized by cell

lysis. High titer viral stocks were grown from individual plaque isolates of each recombinant virus by infection of NIH-3T3 mouse fibroblasts. The BclI-BglI source for each recombinant virus was confirmed by MspI (HpaII) size analysis of Hirt isolated DNA. Each recombinant virus is thus denoted as A2(X), where 'X' designates the viral source of the BclI-BglI small fragments.

Sequence analysis of the recombinant viruses:

Hirt DNA from each of the recombinant viruses was isolated from infected NIH-3T3 mouse cells and digested with BamHI and EcoRI in the cases of the viruses A2(A8), A2(A9), A2(SD-15), and A2(NG-23), while A2(II-5) Hirt DNA was cleaved with HindIII and BamHI (since the BclI-BglI small fragment of the hr-t virus II-5 contains an additional EcoRI site, data not shown).

The approximately 2200 bp fragments were purified by low melting temperature agarose electrophoresis and ligated to the bacterial plasmid pAT153 digested with the appropriate enzymes to allow directional subcloning. Ligated DNA's were used to transform competant DH5 $dam^{-}dcm^{-}$ E.Coli, and ampicillin resistant, tetracycline sensitive clones were isolated. Clones containing proper inserts were confirmed by restriction enzyme analysis of miniprep DNA, and large scale DNA preparations were made. These

plasmids are called pAT:A2(X), where 'A2(X)' designates the source of the polyoma DNA in each plasmid.

Each pAT:A2(X) plasmid was digested with BclI and PstI to release the enhancer containing 600-700 bp fragments. These fragments were purified by low melting temperature agarose electrophoresis and ligated into the polylinker region of either of the bacteriophage M13mp18 or M13mp19 RF DNA's digested with BamHI and PstI. Insert containing clones were isolated, single stranded DNA of each was isolated, and both strands of each recombinant virus were subjected to Sanger dideoxy sequencing from opposite directions by using the M13 universal primer or a 17mer on the early side of the polyoma BglI site.

Test of the replication properties of the recombinant viruses:

NIH-3T3 cells were seeded onto 60mm dishes at a density of $2x10^5$ cells and coinfected with the hr-t mutant virus B-2 at an MOI of 5, and each A2(X) virus at a MOI of 0.5. Individual infections were also carried out with each A2(X) virus alone at an MOI of 0.5. Input ratios were verified 4 hours post-infection via Hirt extractions (data not shown). PCC4 undifferentiated embryonal carcinoma cells were kindly provided by A. Levine, infected with each of the A2(X) viruses at an MOI of 10, and DNA was isolated as above. All cells were grown in Dulbecco's

Figure 1. BclI-BglI size analysis of the A2(X) viruses.

Approximately three micrograms of each of the pAT:A2(X) plasmids were digested with BclI and BglI in the appropriate reaction conditions, electrophoresed through a 2% agarose gel, and the digestion products were visualized by ethidium bromide staining of the gel. The arrow indicates where the various BclI-BglI enhancer containing fragments of each A2(X) virus migrate in relation to strain A2. The source of each BclI-BglI fragment is as follows: Lane 2=A2(A8), Lane 3=A2(A9), Lane 4=A2(SD-15), Lane 5=A2(II-5), Lane 6=A2(NG-23), Lane 7=A2. Lane 1 is a size marker (Lamda DNA digested with Hind III).

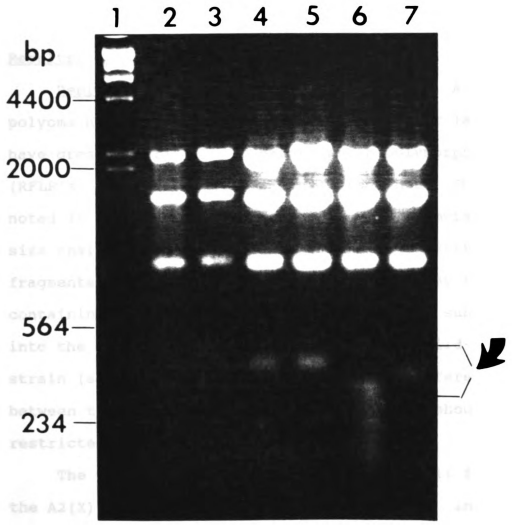


Figure 1. BclI-BglI size analysis of the A2(X) viruses.

modified Eagle's medium supplemented with 10% calf serum (fetal calf serum for PCC4 EC cells) and antibiotics.

Results:

Replicative abilities of A2(X) viruses: A number of polyoma hr-t mutant viruses maintained in our laboratory have gross restriction fragment length polymorphisms (RFLP's) in their small BclI-BglI fragments. This had been noted in the original isolation of the hr-t mutants because size analyses of their DNA had demonstrated RFLP's in MspII fragments #3 and #5.[13,39] To analyze and test the enhancer containing regions of these hr-t mutants, we subcloned them into the genomic BclI-BglI fragment of the wild-type A2 strain (see Methods). The only sequence differences between these reconstructed 'A2(X)' viruses should be restricted to their non-coding regions.

The various RFLP's in the small BclI-BglI fragments of the A2(X) viruses are demonstrated in Fig.1. In comparison to the same region in wild type strain A2, the A2(A8) and A2(NG-23) viruses have deletions of approximately 20 bp, A2(SD-15) and A2(II-5) have additions of approximately 40 bp, (with A2(II-5) also possessing an EcoRI site within this region) and A2(A9) an addition of approximately 100 bp.

Figure 2. Replication of the A2(X) viruses in comparison to A2, or in competition with B-2.

NIH-3T3 cells were infected with each virus alone at an MOI of 0.5 ("-" lanes) or in a competition assay in which the MOI ratio of A2(X):B-2 was 0.5:5 ("+" lanes). At 60 hours post infection viral DNA was isolated by Hirt extraction, 1/50 of each sample was digested with the restriction enzyme PvuII and electrophoresed through a 1.6% agarose gel. DNA was transferred to nitrocellulose and hybridized to a probe specific for the polyoma Hpa II-#4 fragment (B-2 contains a 240 bp deletion in this fragment). Viruses used in each infection are as follows: 1= A2, 2= A2(A8), 3=A2(A9), 4=A2(SD-15), 5=A2(II-5), 6=A2(NG-23).

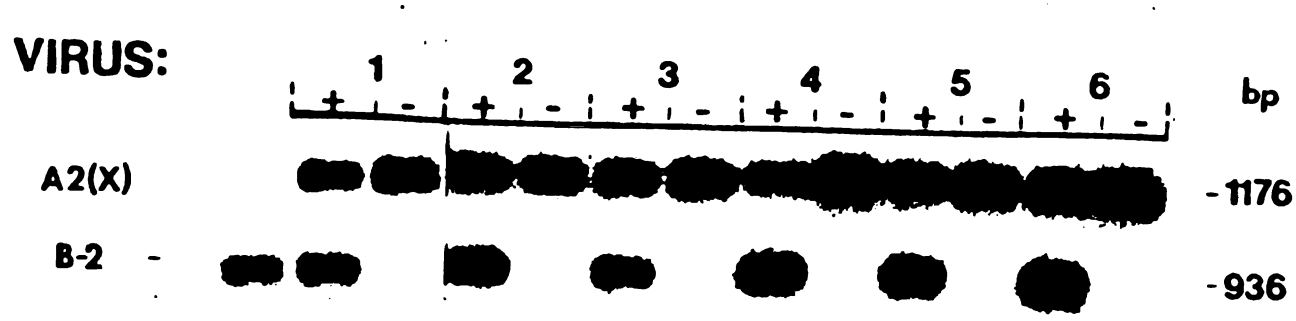


Figure 2. Replication of the A2(X) viruses in comparison to A2, or in competition with B-2.

To assess the ability of each recombinant virus to replicate in permissive mouse cells, NIH-3T3 cells were infected with either a recombinant virus alone, or coinfected with the recombinant virus along with the hr-t mutant B-2. This competition assay was devised in our lab to detect subtle differences in the ability of viral strains to replicate (see Figure 2). It is readily apparent that all of the recombinant viruses replicate to levels equivalent to the A2 strain of virus, either in single infections or when forced to compete with B-2, even when B-2 was initially introduced in a 10 fold excess over the A2(X) viruses in the initial infection mix. The intensity of every band on this blot is within 2.5 fold of any other based on densitometric scanning, indicating that the A2(X) viruses all replicate to wild type A2 levels, and they do not significantly differ in their ability to replicate in NIH-3T3 cells. These results have been obtained in three independent experiments using different multiplicities of infection, and is indirectly verified by the similar titers of infectious virus obtained when these viruses are grown in NIH-3T3 cells (data not shown).

Sequence analysis of the A2(X) viruses: We sequenced both strands of the BclI-BglI region of each of the A2(X) viruses by subcloning into the bacteriophage M13 and utilizing the Sanger dideoxy chain termination method (see Methods). The sequencing results are described

Figure 3. Sequence analysis of the A2(X) viruses.

The sequence of each of the A2(X) viruses as well as our A2 strain are compared to a restriction map of the A2 strain of Salzman (35). The strand equivalent to the early mRNA is presented. Dark parallel lines represent duplications and dashed lines indicate their relation to the genome. Nucleotide substitutions are placed directly above their location, with nucleotide additions placed over a "+", and nucleotide deletions placed over a "-". Boundries of the major duplications are placed in relation to the nucleotide numbers of the A2 schematic diagram.

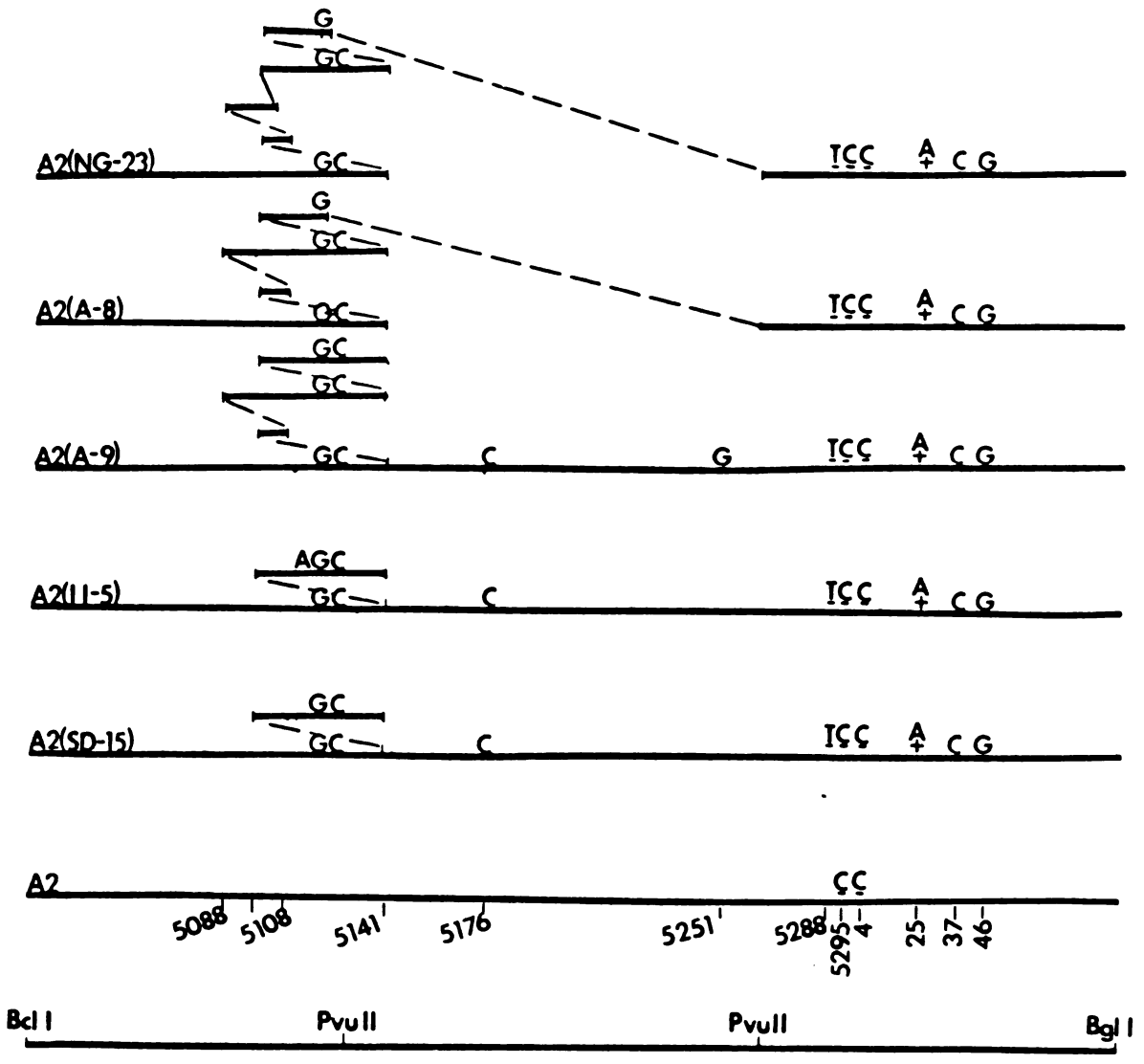


Figure 3. Sequence analysis of the A2(X) viruses.

schematically in Figure 3. All the A2(X) viruses contain at least a single duplication of the previously described A transcriptional enhancer element[14], a point followed up in the discussion.

Briefly, the recombinant viruses A2(SD-15) and A2(II-5) demonstrate 44 bp duplications identical to those described previously for the P16 and Toronto strains of polyoma, respectively.[33,34] However, the three remaining recombinants have multiple and more extensive duplications of the region only duplicated once in the A2(SD-15) and A2(II-5) viruses. In strains A2(A8) and A2(NG-23) this amplification is coupled with a deletion of 123 bp between nucleotides 5141 and 5264. Since all of these recombinants are able to replicate to wild type levels in mouse NIH-3T3 cells, the 123 bp deleted sequences are not absolutely required for viral replication in these cells.

These sequence rearrangements are particularly striking when they are compared to the sequence variations of some host range mutants of polyoma selected to grow on the normally non-permissive undifferentiated PCC4 embryonal carcinoma cell line.[18,25] The enhancer regions of the PCC4 variants 204 and 97 are shown in comparison to the enhancer regions of the A2(A8) and A2(NG-23) viruses (see Figure 4). The viruses compared in Figure 4 all have

Figure 4. Comparison of A2(A8) and A2(NG-23) to the PCC4 EC mutants.

The enhancer region of the viruses A2(A8) and A2(NG-23) are compared to the same region in the embryonal carcinoma mutants PCC4-97 and PCC4-204. Regions suggested to be required of all PCC4 mutants by Blangy, et al.[25] and the "omega" element, as described by Amati, et al.[23] are schematically presented also.

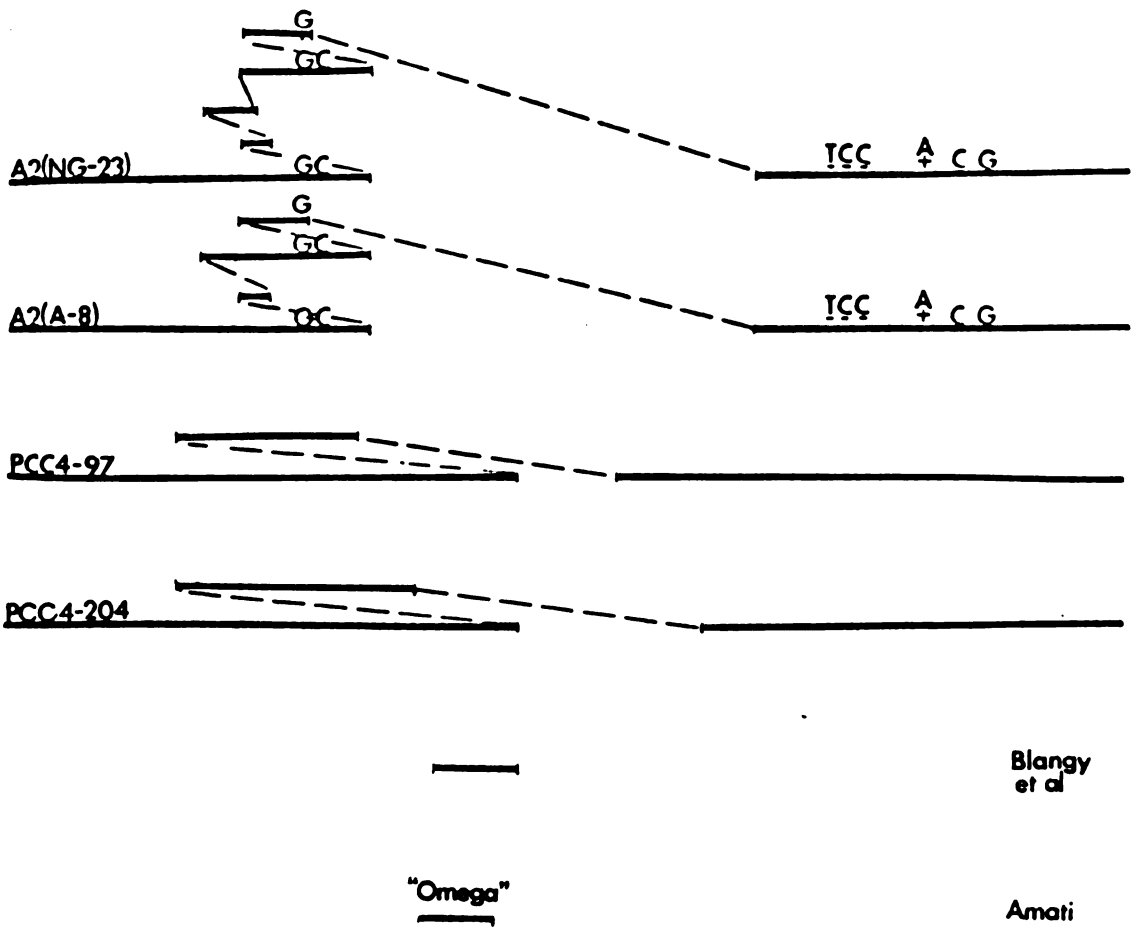


Figure 4. Comparison of A2(A8) and A2(NG-23) to the PCC4 EC mutants.

duplications coupled with deletions, however A2(A8) and A2(NG-23) have a deletion which is larger than that of the polyoma PCC4 mutants. The larger deletion includes nucleotides 5173-5200, a region previously suggested to be required in all polyoma mutants able to grow on undifferentiated PCC4 cells isolated to date.[25] We infected undifferentiated PCC4 cells with each of the reconstructed viruses, and assessed their ability to replicate (see Figure 5). Wild type strain A2 replicated to very low levels, and none of the constructed viruses were able to replicate to levels any greater than that observed for wild type A2 strain, suggesting that the 5173-5200 bp region of polyoma is required for replication in undifferentiated PCC4 cells, (in addition to the duplications and deletions already observed in the PCC4 mutants isolated to date).[45]

Discussion

Previous studies have defined the polyoma BclI-PvuII 242 bp fragment as a transcriptional enhancer element by its ability to increase transcription from a distant promoter in an orientation independent manner.[8] Numerous studies have dissected the transcriptional enhancer into functionally redundant subdomains,[12,14,26] as summarized in Figure 6. Those domains, designated as 'transcriptional

Figure 5. Replication of the A2(X) viruses in PCC4 EC cells.

PCC4 cells were infected at an MOI of 10 with each of the A2(X) viruses or A2. 1/4 of the Hirt extractable viral DNA was isolated from each plate at 3 or 72 hours post infection, digested with the restriction enzyme BamHI, and electrophoresed in a 0.7% agarose gel. DNA was transferred to nitrocellulose and hybridized to a probe specific for the entire polyoma genome. Viruses used are as follows: a=A2, b=A2(A8), c=A2(A9), d=A2(SD-15), e=A2(II-5) and f=A2(NG-23).

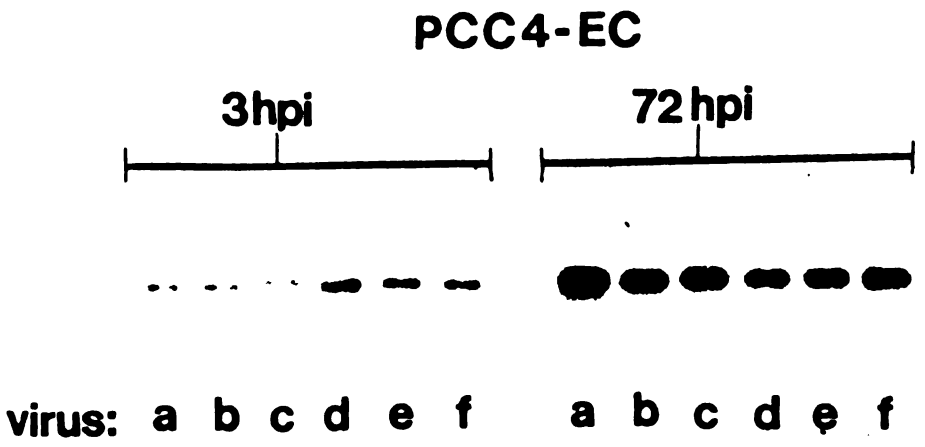


Figure 5. Replication of the A2(X) viruses in PCC4 EC cells.

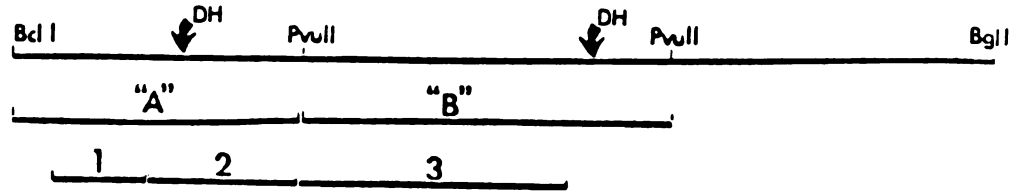
enhancer elements', have also been demonstrated to be required for viral DNA replication.[12,22,42,44,48] We have studied rearrangements of the non-coding region of viable viruses constructed in this study (see Figure 6) and how they relate to these enhancer elements.

Most striking is the frequency in which polyoma variants possess duplications of subdomains of the enhancer region.[1] Indeed, all five viruses investigated in this study have at least a single duplication of sequences encompassing the 'A', or 'alpha core' domains. These same sequences have been demonstrated to be responsive to the middle T protein of polyoma.[50] Since these enhancers were originally isolated from hr-t mutants with defective middle T antigens,[2,39] the duplications may have arisen in order to increase interactions with middle T responsive trans-acting transcriptional factors (which bind to middle T responsive sequences within the duplicated region).[49,50]

It is interesting to note that each rearranged region contains a G to C transversion at nt 5117 and an A to G transition at nucleotide 5123. These changes have been observed in other previously sequenced strains of polyoma, notably Toronto and P16.[34] The transversion at 5117 is particularly interesting because it has been demonstrated that a non-functional enhancer sequence (B122), which contains multiple point mutations, can return to wild type

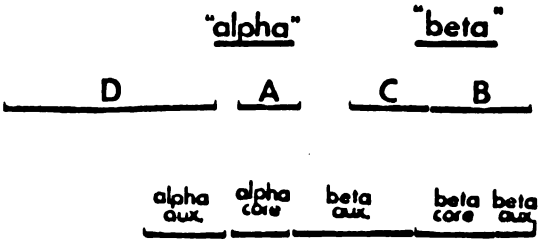
Figure 6. The transcriptional/replicational enhancer elements, and the factors which bind to this region.

The boundaries of the transcriptional and replicational enhancer element boundaries, as described by others,[14,15,27,28,48] are compared. The DNA binding sites of trans-acting factors which have been demonstrated to bind to this region are also presented. These features are all compared to the physical map of A2(NG-23). In the figure, DH=DNase hypersensitive site, and filled in triangles=inverted repeat region.



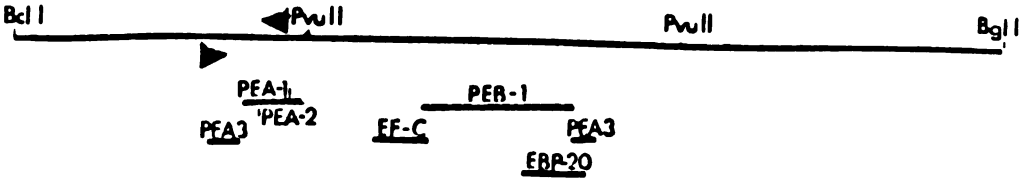
TRANSCRIPTIONAL

ENHANCER ELEMENTS

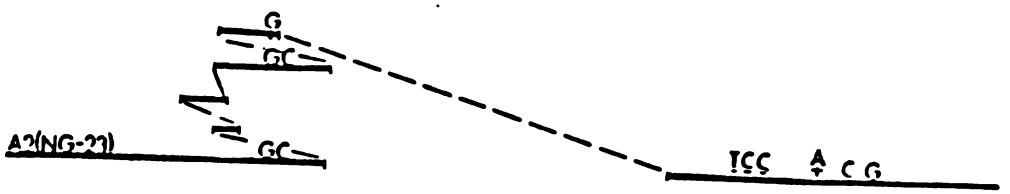


REPLICATIONAL

ENHANCER ELEMENTS



FACTORS



levels of function with only a single reversion at nt 5117.[42] In addition, the positive trans-acting factor PEA1, binds to nucleotides 5115-5125,[24,31] a binding which can be specifically blocked by mutations at nucleotides 5116 and 5118.[32] Possibly, these duplicated regions increase the ability of polyomaviruses to interact with PEA1, or PEA1 like factors.

Interestingly, there is an inverted repeat sequence in the polyoma genome at nucleotides 5120-5127 and 5099-5106 (see filled in triangles, Figure 6). In fact, this sequence is part of the recognition sites for two different factors, PEA1 and PEA2.[32] The nucleotides making up the 5099-5106 bp portion of the inverted repeat are duplicated in all of the reconstructed viruses, (without the A to G transition present at nucleotide 5123, within the right hand portion of the inverted repeat), up to four times in the enhancers of A2(A8), A2(A9), and A2(NG-23), suggesting that the sequences at 5099-5106 may be functionally important, possibly by binding trans-acting factors similar to PEA1 and PEA2.

A third feature of the reconstructed viruses is that the location of their duplications always center about one of the two known DNaseI hypersensitive sites mapped to nucleotide 5100.[15] This region may be more sensitive to duplication since it is relatively nucleosome free, suggesting that the naked DNA might present a more

susceptible substrate for the recombination machinery of the host cell. This site is located within the A transcriptional enhancer, the dominant enhancer element in mouse cells.[14] Together, these features may favor selection of variant genomes which possess these duplicated regions. In the most extreme examples of amplification, the rearrangements of A2(A8) and A2(NG-23), a simultaneous deletion of the less dominant B enhancer element[14] has also occurred.

The results of Figure 2 clearly demonstrate that each of the reconstructed viruses can replicate to wild type levels in the mouse NIH-3T3 cell line. A2(A8) and A2(NG-23) can replicate to wild type levels, despite deletions of sequences equivalent to the C replicational domain[48] or 'Omega' element,[23] (nt 5159-5183, see Figure 6), providing the first documentation of viable polyoma viruses deficient in this element. It has been previously suggested the the 'omega element' is absolutely required for viability, since no viral isolate (normal or host range mutant) has ever been reported to lack this domain.[23] Indeed, a factor called EF-C was found to bind this sequence in a number of cell lines, including mouse L cells, implicating it as a DNA binding protein pivotal to polyoma replication.[29] The multiple duplications of the A enhancer domain present in the viruses A2(A8) and A2(NG-23) may have allowed for the subsequent loss of the C domain.

A number of host range mutants have been isolated based upon their ability to grow in cells normally non-permissive for polyomavirus replication.[1] It was shown that these mutants were selected due to their possession of rearranged enhancer subdomains.[1] The most relevant comparison of the A2(X) enhancer rearrangements is to those of the host range mutants which grow in the undifferentiated embryonal carcinoma (EC) cell lines. The EC cells are non-permissive for polyoma unless they are first induced to differentiate. Polyoma mutants which replicate in the F9 EC cell line have at least a single bp change at nucleotide 5235 (or duplication encompassing this bp change) responsible for their new host range [10,11,36,43] Polyoma host range mutants selected on the PCC4 EC cell line have more extensive modifications responsible for their new host range, most commonly a duplication in the A transcriptional enhancer domain coupled with a deletion in the B transcriptional enhancer domain.[25,38,45,46] Trans-acting factors bind only weakly to the A transcriptional enhancer domain in undifferentiated EC cells, while binding is easily demonstrated upon differentiation.[21] The existence of polyoma PCC4 EC mutants suggests that duplication of this region may allow for increased interaction with these factors, which correlates with the new host range. However, our results demonstrating lack of replication in undifferentiated PCC4 cells of the viruses

A2(SD-15), A2(II-5), and especially A2(A9) (see Figure 5) argue against this being the only requirement for growth in EC cells, as has previously been noted for other viral strains with duplications of this region.[25]

Others have suggested that the deletions in the B transcriptional enhancer domain prevents polyoma host range mutants from interacting with negatively acting factors present in the undifferentiated EC cells, which then allows for viral replication. Labile proteins, as well as E1a like repressor activities have been suggested to be responsible for the replicational blockades, possibly by binding to sequences deleted in the PCC4 host range mutants[4,5,47] However, these deletions have never been demonstrated to extend into the C domain of any polyoma host range mutant to date, suggesting that this domain is crucial for viral replication in undifferentiated PCC4 EC cells.[25] The viruses A2(A8) and A2(NG-23) have enhancer region rearrangements which are strikingly similar to the rearrangements of known PCC4 EC mutants (each possessing extensive duplications of the A transcriptional enhancer domain concommitant with deletions in the B transcriptional enhancer domain) with the exception that they have a larger deletion in the region which includes the C transcriptional enhancer domain. Their lack of replication in undifferentiated PCC4 EC cells supports the hypothesis that the C domain is required for growth of polyoma viruses in

PCC4 EC cells, even when duplications of the A transcriptional enhancer domain coupled with deletions in the B transcriptional enhancer are present in the same genome.

In summary, we have constructed a number of viable polyoma viruses which all have multiple enhancer region rearrangements. The actual boundaries of these rearrangements allowed us to conclude that the previously described omega element is not absolutely required for replication of polyomavirus, at least in mouse NIH-3T3 cells. However, the results did suggest that the enhancer sequences between nucleotides 5159-5183 are required for replication of polyomavirus in PCC4 EC cells (as had been previously suggested[25]). Finally, we are currently investigating the ability of each of these viruses to infect neonatal mice, to ascertain whether the same regions implicated as important in the tissue culture systems used in this study are also relevent in actual infections of polyomas natural host. The complexity of these interactions underscores the highly regulated nature of mammalian gene expression and replication.

REFERENCES

1. Amati, P. (1985). Polyoma regulatory region: A potential probe for mouse cell differentiation. Cell 43: 561-562.

2. Benjamin, T.L. (1970). Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. USA 67: 394-399.

3. Bohnlein, E., Gruss, P. (1986). Interaction of distinct nuclear proteins with sequences controlling the expression of polyomavirus early genes. Mol. Cell. Biol. 6:1401-1411.

4. Borrelli, E., Hen, R., Chambon, P. (1984). Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature (London) 312:608-612.

5. Cremisi, C., Babinet, C. (1986). Negative regulation of early polyomavirus expression in mouse embryonal carcinoma cells. J. Virology 59:761-763.

6. Dandolo, L., Blangy, D., Kamen, R. (1983) Regulation of polyoma virus transcription in murine embryonal carcinoma cells. J. Virology 47:55-64

7. DeSimone, V., Amati, P. (1987). Replicative cis-advantage of polyomavirus regulatory region mutants in different murine cell lines. J. Virology 61:1615-1620.

8. deVilliers, J., Schaffner, W. (1981) A small segment of polyoma virus DNA enhances the expression of a cloned beta-globin gene over a distance of 1400 base pairs. Nucleic Acids Research 9:6251-6264.

9. deVilliers, J., Schaffner, W., Tyndall, C., Lupton, S., Kamen, R. (1984). Polyoma virus DNA replication requires an enhancer. Nature (London) 312:242-246.

10. Fujimura, F.K., Deininger, P.L., Friedmann, T., Linney, E. (1981). Mutation near the polyoma DNA replication origin permits productive infection of F9 embryonal carcinoma cells. Cell 23: 809-814.

11. Fujimura, F.K., Silbert, P.E., Eckhart, W., Linney, E. (1981). Polyoma virus infection of retinoic acid-induced differentiated teratocarcinoma cells. J. Virology 39: 306-312.

12. Hassell, J.A., Muller, W.J., Mueller, C.R. (1984). The dual role of the polyomavirus enhancer in transcription and DNA replication. Cancer Cells 4: DNA tumor viruses, 561-569.

13. Hattori, J., Carmichael, G.G., Benjamin, T.L. (1979). DNA sequence alterations in hr-t deletion mutants of polyoma virus. Cell 16: 505-513.

14. Herbomel, P., Bourachot, B., Yaniv, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39: 653-662.

15. Herbomel, P., Saragosti, S., Yaniv, M. (1981). Fine structure of the origin-proximal DNase I hypersensitive region in wild-type and EC mutant DNA. Cell 45:461-470.

16. Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365-369.

17. Johnson, P.F., Landschulz, W.H., Graves, B.J., McKnight, S.L. (1987). Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes and Development 1:133-146.

18. Katinka, M., Vasseur, M., Montreau, N., Yaniv, M., Blangy, D. (1981). Polyoma DNA sequences involved in control of viral gene expression in murine embryonal carcinoma cells. Nature (London) 290:720-722.

19. Katinka, M., Yaniv, M., Vasseur, M., Blangy, D. (1980). Expression of polyoma early functions in mouse embryonal carcinoma cells depends on sequence rearrangements in the beginning of the late region. Cell 20:393-399.

20. Kovesdi, I., Satake, M., Furukawa, K., Reichel, R., Ito, Y., Nevins, J.R. (1987). A factor discriminating between the wild-type and a mutant polyomavirus enhancer. Nature (London) 328:87-89.

21. Kryszke, M.H., Piette, J., Yaniv, M. (1987). Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. Nature (London) 328:254-256.

22. Luthman, H., Nilsson, M.G., Magnusson, G. (1982). Non-contiguous segments of the polyoma genome required in cis for DNA replication. J. Mol. Biol. 161:533-550.

23. Maione, R., Passananti, C., De Simone, V., Delli-Bovi, P., Augusti-Tocco, G., Amati, P. (1985). Selection of mouse neuroblastoma cell-specific polyoma virus mutants with stage differentiative advantages of replication. EMBO 4: 3215-3221.

24. Martin, M.E., Piette, J., Yaniv, M. Tang, W.J., Folk, W.R. (1988). Activation of the polyomavirus enhancer by a murine activator protein 1 (AP1) homolog and two contiguous proteins. Proc. Natl. Acad. Sci. USA 85:5839-5843.

25. Melin, F., Pinon, H., Reiss, C., Kress, C., Montreau, N., Blangy, D. (1985) Common features of polyomavirus mutants selected on PCC4 embryonal carcinoma cells. EMBO 4: 1799-1803.

26. Mueller, C.R., Mes-Masson, A.M., Bouvier, M., Hassell, J.A. (1984). Location of sequences in polyomavirus DNA that are required for early gene expression in vivo and in vitro. Mol. Cell. Biol. 4:2594-2609.

27. Muller, W.J., Dufort, D., Hassell, J. (1988). Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. Mol. Cell. Biol. 8:5000-5015.

28. Muller, W.J., Mueller, C.R., Mes, A.M., Hassell, J.A. (1983). Polyomavirus origin for DNA replication comprises multiple genetic elements. J. Virology 47: 586-599.

29. Ostapchuk, P., Diffley, J.F.X., Bruder, J.T., Stillman, B., Levine, A.J., Hearing, P. (1986). Interaction of a nuclear factor with the polyomavirus enhancer region. Proc. Natl. Acad. Sci. USA 83:8550-8554.

30. Piette, J., Kryszke, M.H., Yaniv, M. (1985). Specific interaction of cellular factors with the B enhancer of polyoma virus. EMBO 4:2675-2685.

31. Piette, J., Yaniv, M. (1986). Molecular analysis of the interaction between an enhancer binding factor and its DNA target. Nucl. Acids Res. 14:9595-9611.

32. Piette, J., Yaniv, M. (1987). Two different factors bind to the alpha domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. EMBO 6:1331-1337.

33. Rothwell, V.M., Folk, W.R. (1983). Comparison of the DNA sequence of the Crawford small-plaque variant of polyomavirus with those of polyomaviruses A2 and strain 3. J. Virology 48:472-480.

34. Ruley, H.E., Fried, M. (1983). Sequence repeats in a polyoma virus DNA region important for gene expression. J. Virology 47:233-237.

35. Salzman, N.P. ed. (1986). The papovaviridiae. Volume I: The Polyomaviruses. Plenum Press, New York and London.

36. Satake, M., Furukawa, K., Ito, Y. (1988). Biological activities of oligonucleotides spanning the F9 point mutation within the enhancer region of polyomavirus DNA. J. Virology 62:970-977.

37. Scholer, H.R., Gruss, P. (1984). Specific interactions between enhancer-containing molecules and cellular components. Cell 36:403-411.

38. Sekikawa, K., and Levine, A.J. (1981). Isolation and characterization of polyoma host range mutants that replicate in nullipotential embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA. 78:1100-1104.

39. Staneloni, R.J., Fluck, M.M., Benjamin, T.L. (1977). Host range selection of transformation-defective hr-t mutants of polyoma virus. Virology 77:598-609.

40. Struhl, K., (1985). A rapid method for creating recombinant DNA molecules. Biofeedback (3) 452-453.

41. Tanaka, K., Chowdhury, K., Chang, K.S.S., Israel, M., Ito,Y. (1982). Isolation and characterization of polyoma virus mutants which grow in murine embryonal carcinoma and trophoblast cells. EMBO 1:1521-1527.

42. Tang. W.J., Berger, S.L., Triezenberg, S.J., Folk, W.R. (1987). Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. Mol. Cell. Biol. 7:1681-1690.

43. Tseng, R.W., Williams, T., Fujimura, F. (1988). Unique requirement for the Py F441 mutation for polyomavirus infection of F9 embryonal carcinoma cells. J. Virology 62:2896-2902.

44. Tyndall, C., LaMantia, G., Thacker, C.M., Favaloro, J., Kamen, R. (1981). A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nuc. Acids Res. 9:6231-6250.

45. Vasseur, M., Katinka, M., Herbomel, P.J., Yaniv, M., Blangy, D. (1982). Physical and biological features of polyoma virus mutants able to infect embryonal carcinoma cell lines. J. Virology 43:800-808.

46. Vasseur, M., Kress, C., Montreau, N., Blangy, D. (1980). Isolation and characterization of polyoma virus mutants able to develop in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 77: 1068-1072.

47. Velcich, A., Kern, F.G., Basilico, C., Ziff, E.B. (1986). Adenovirus E1a proteins repress expression from polyomavirus early and late promoters. Mol. Cell. Biol. 6: 4019-4025.

48. Veldman, G.M., Lupton, S. Kamen, R. (1985). Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. 5:649-658.

49. Wasylyk, B., Imler, J.L., Chatton, B., Schatz, C., Wasylyk, C. (1988). Negative and positive factors determine the activity of the polyoma virus enhancer alpha domain in undifferentiated and differentiated cell types. Proc. Natl,. Acad. Sci. USA 85:7952-7956.

50. Wasylyk, C., Imler, J.l., Wasylyk, B. (1988). Transforming but not immortalizing oncogenes activate the transcription factor PEA1. EMBO 7: 2475-2483.

Chapter 3

Defining subdomains of the polyoma enhancer region required for high levels of viral replication and persistence in mouse tissues.

ABSTRACT

The ability of polyoma virus variants to replicate in mouse tissues is described, utilizing a modified whole body in-situ hybridization technique and extraction of viral DNA from individual organs. Wild-type A2 strain polyomaviruses carrying enhancer regions from hr-t mutants were tested. Specifically, the viruses A2(A8) and A2(NG-23) have deletions of 123 bp (which contains a subdomain not required for replication mouse NIH-3T3 fibroblasts), responsible for a 30-fold decrease in the ability of these viruses to replicate in neonatal mouse tissues when compared to a wild type strain. Another virus, A2(A9), can replicate to wild type levels early in infection but cannot persist to wild type levels in 4 week old kidney tissues. This suggests that viral persistence is not linked to high levels of replication early in infection.

Introduction

Polyoma virus was initially discovered as a contaminating source of parotid tumors in cell-free extracts of leukemic Ak mice.[16] The agent was shown to be a virus able to elicit a plethora of tumors including tumors of the salivary glands, mammary tumors, epithelial thymomas, and hair follicle tumors.[2,3,5,18,41] Over the years well over 30 different types of tumors have been described.[6] This ability to induce tumors in such a vast number of distinct mouse tissues, epithelial as well as mesenchmyal, suggests that the virus has been under selective pressure to interact with ubiquitous factors/pathways present in all of these tissues.[5] Because of their small size, polyomavirus as well as the closely related SV40 have been used extensively as probes to understand mammalian gene expression and replication.

Early studies demonstrated that the highest levels of infectious virus production is always observed in neonatally infected mice.[37] Infectious virus was isolated at the highest levels in the kidneys, with salivary gland, liver, lung, and spleen harboring intermediate levels, and brain and blood containing the lowest levels of infectious virus.[38]

More recent work based on a modified tissue transfer technique and isolation of polyoma DNA from infected neonate tissues has confirmed and refined these early findings.[10,11] It was demonstrated that in neonate mice infected intra-peritoneally (i.p.) with the wild type A2 strain, a systemic infection develops which includes the lungs, liver, spleen, and kidneys, with replication levels peaking at 7-8 days post infection (dpi), and then decreasing as neutralizing antibody titers rise.[10,11] As had been demonstrated previously,[25,28] the virus was found to persist in kidney tissue well beyond clearance by the immune response.[11] Interestingly, when the neonates were inoculated inta-nasally, the lungs also become a site of viral persistence.[11]

It is not clear, however, what determinants of the virus actually control tissue tropism in mouse infections. The wide range of cell types displaying tumors and/or lytic damage indirectly suggests that the virus receptor and host factors controlling viral gene expression are ubiquitous.

It might be anticipated that the enhancer region could play a role in tropism. However, studies with natural isolates which only differ in the enhancer region have not demonstrated any significant changes in the tumor pattern elicited by these viruses.[2] In a more artificial construct, a virus whose B enhancer domain was substituted with the LTR sequence from the Moloney murine leukemia

virus demonstrated a profound alteration in tissue tropism, as evidenced by replication in pancreatic tissue, a tissue that is not normally a site of viral replication[36] Furthermore, non-coding sequences outside of the enhancer on the early side of the replication origin have been shown to specifically control epithelial thymoma formation.[6,12,13]

In tissue culture systems it is clear that the enhancer region is directly involved in modulating the replicative host range of the virus.[1] This region is composed of multiple, functionally redundant subdomains, required not only as transcriptional enhancers, but also as replicational enhancer sequences.[9,19,20,26,30-32,43,44,46] A number of host range mutants have been shown to undergo rearrangements of their enhancer regions to allow replication in normally non-permissive cell lines.[1] Some of these cell lines include embryonal carcinoma, trophoblastic, neuroblastic, and Friend leukemic cell lines.[7,14,22,27,29,42,45] In some studies actual interaction with cell line specific factors has been correlated with the new replicative potential.[23,24,47]

We have undertaken to test a collection of viable mutant strains which only differ in their enhancer regions. The derivation and properties of these isolates in tissue culture systems along with their enhancer rearrangements is described in Chapter 2. In the present report we will

describe an investigation into the replicative abilities of these viral strains in infections of neonate Balb/c mice using a modified whole body in-situ hybridization technique. Our results suggest that various alterations of the enhancer affect replication in mouse tissues. In particular, a subdomain of the polyoma enhancer not required for growth of the virus in mouse fibroblast tissue culture cell lines is required for maximal replication in the mouse.

Materials and Methods

Construction and isolation of polyoma mutants:

We have previously described the construction of the A2(X) mutants. Briefly, the enhancer containing BclI-BglI small fragment from the hr-t mutant strains A8, A9, SD-15, II-5, and NG-23 were subcloned into the BclI-BglI large fragment of strain A2. These recombinants were all viable when grown on NIH-3T3 cells. Stocks of equivalent high titer were produced, as determined by plaque assay on NIH-3T3 cells.

In-situ hybridization of whole mouse sections:

Balb/c pups, less than 24 hours old, were each injected i.p. with 50 uL of the appropriate virus stock, (either one of the A2(X) viruses or wild type strain A2)

all at $2.4x10^7$ pfu/mL. At the times indicated, mice were sacrificed by etherization and embedded into a block of 3% carboxy-methylcellulose by freezing in liquid nitrogen gas. Blocks could be stored at -70°C for several months without significant loss of hybridizeable signals. Blocks were sectioned to an appropriate sagittal plane with a LKB #2258 cryomicrotome, and then 40 micron sections were transferred to 3M tape #845. Sections were air dried for one minute, fixed in a 3:1 mixture of ethanol/acetic acid for 20 minutes at room temperature, dehydrated twice in 95% ethanol for 5 minutes, air dried and stored at room temperature. The ethanol/acetic acid fixation proved to be the superior fixative with regards to allowing for maximal hybridization with minimal loss of tissue morphology.[17] Fixed sections could also be stored several months at room temperature without significant loss of signal.

After fixation and drying, sections were denatured at 65°C in a preheated solution of 95% deionized formamide in 0.1x SSPE for 20 minutes, and then plunged into ice-cold 0.1x SSPE, (1x SSPE is 0.15M NaCl, 0.01M $NaH_2PO_4.H_2O$, and 0.1mM EDTA pH=8.0).

Hybridization was carried out as described,[40] except that SSPE was substituted for SSC, which seems to decrease, but not eliminate, non-specific binding of ^{32}P labelled probes to bone tissue (especially a problem in older mice). Tape with sections were placed into Seal-A-Meal bags with 3

mL of prehybridization solution per section and incubated at 37°C for 18-24 hours. Prehybridization solution (PS) is: 50% deionized formamide, 5x SSPE, 2.5x Denhardt's solution (1x Denhardt's is 0.02% bovine serum albumin, 0.02% ficoll, and 0.02% polyvinylpyrrolidine) with 100 ug/ml of boiled, sonicated, salmon sperm DNA. Hybridization to the denatured sections was carried out at 37°C for 66-72 hours with 1 mL per section of PS and 0.5-1x10^6 cpm of polyoma specific whole genome probe, (probe was random primed, column purified, and always of specific activities greater than 1x10^9cpm/ug). Hybridized sections were washed three times for 30 minutes in 2x SSPE at 37°C, once for 30 minutes in 2x SSPE at 65°C, and finally for 30 minutes in 0.1x SSPE at 65°C. Sections were then stained in haemotoxylin for one minute, rinsed in H_2O, washed in 1% Li_2CO_3 for one minute, rinsed again in H_2O, and air dried. This staining protocol limited, but did not eliminate dehydration artifacts in the sections. Finally, sections were adhered to transparent sheets and exposed to Kodak X-Omat film with intensifier screens for 24-96 hours at -70°C. Developed films were directly compared to the stained section to identify the tissues containing viral-specific signals.

Tissue extractions: Mice were sacrificed and the appropriate organs were removed and either stored in liquid nitrogen for later processing, or placed directly into 1

mL/mg "tissue digestion buffer' (tissue digestion buffer is: 100 mM NaCl, 10 mM Tris pH=8, 25 mM EDTA pH=8, 0.5% SDS, and proteinase K at 0.1 mg/mL). Tissue was homogenized at 30,000 rpm with a Tekmar tissuemizer and digested at 50°C for 12-18 hours with shaking. Digested tissue was extracted with phenol/chloroform, treated with RNase A (1 ug/mL, at 37°C for 1 hour) reextracted with phenol/chloroform, then chloroform. Total DNA was precipitated by addition of 1/10 volume 3M NaAcetate and two volumes of 95% ethanol, mixed, and stored overnight at -20°C. DNA was collected by centrifugation at 10,000 rpm in a Sorvall SA-600 rotor, resuspended in 10 mM Tris, 1 mM EDTA, and the final DNA concentration was measured by absorbance at 260 nM. Digestions with restriction endonucleases were as described in the figure legends using the appropriate reaction conditions.

Results

In-situ hybridization of mouse sections: To facilitate the detection of DNA by hybridization in tissue sections, we modified a previously described in-situ hybridization technique originally derived to detect viral RNA.[40] The specificity of the hybridization conditions is demonstrated in Figure 1. Probes generated from random DNA sequences did not hybridize to any sections (data not shown). The fact that even without denaturation

Figure 1. In-situ hybridization to control sections.

Adjacent sections were taken from an A2 infected mouse and fixed as described in Methods. Section numbers 1, 2, and 7 were not denatured prior to hybridization with the polyoma specific probe, allowing only for hybridization to RNA sequences.[40] Section 1 was treated with RNase A, and section 7 was treated with RNase and DNase prior to hybridization. Thus, the probe hybridizing to the tissues in sections 1 and 2 is most likely binding to single-stranded DNA, as even extensive RNase A digestion does not eliminate this binding. Sections 3-6 were denatured and hybridized to the polyoma probe. Section 3 was RNase treated, 5 was DNase treated, and 6 was both RNase and DNase treated prior to hybridization. Again, double stranded DNA is being hybridized to, without significant contributions from viral RNA.

Figure 1. In-situ hybridization to control sections.

some polyoma sequences are detected (Figure 1, sections 1 & 2) and the fact that this hybridization is resistant to extensive RNase A digestion, (Figure 1, section 1) suggests that the hybridization conditions facilitate some DNA denaturation, or that some single stranded viral sequences are present. Figure 1 also demonstrates that DNA is the predominant signal, and viral RNA does not significantly hybridize to probe in infected mouse sections. This was true for all sections analyzed. This suggests that either the viral RNA is extremely unstable or at a concentration which is below detectable limits. This protocol allows us to not only detect viral DNA in tissue, but also viral DNA circulating as encapsidated particles. Control sections from non-infected mice of equivalent age were included in all experiments, especially when studying sections of older mice.

While non-specific binding of probe is not a problem for 8 day old mice, (see Fig. 2 and Fig. 3), four week old mice exhibit some non-specific binding, most likely due to increasing mineralization of bone tissue. This problem can be limited by adding SSPE to all hybridization solutions and by extensively purifying away unincorporated radiolabelled nucleotides from the probe (see Methods). As described previously, the technique facilitates visualization and analysis of a number of tissues in a single section.[40]

Figure 2. Time course of A2 replication in mouse tissues visualized by in-situ hybridization.

Neonatal mice were infected with $1.2x10^6$ pfu of wild type A2 virus, sacrificed at the indicated times, and sections were hybridized to polyoma specific probe as described in Methods. Input virus can be detected 12 hours post infection (see arrow) and by 48 hours a substantial increase in viral DNA sequences is detected throughout the abdominal viscera when compared to the 12 hour time point. Two mice per time point are presented.

VIRUS: A2

HOURS POST INFECTION:

Fig. 2 shows a time course of replication when neonatal mice are infected i.p. with $1.2x10^6$ pfu of the wild type A2 strain. The mid-sagittal sections demonstrate that viral replication is reaching high levels by 5 dpi (days post infection) throughout the mouse, consistent with previous studies.[10,11] This experiment also demonstrates that the viral DNA examined by in-situ hybridization at times beyond 5 d.p.i reflects high levels of viral replication, and is not just a reflection of input virus.

Replication of viruses with variant enhancers in neonatal mice: We have previously described the derivation of 5 new viable strains of polyoma virus called the A2(X) viruses. These strains have identical coding regions (that of wild type A2) and differ in the enhancer regions, a region known to affect host range of the virus in tissue culture systems.[1] The enhancer mutations were derived from natural isolates and consist of duplications and deletions of enhancer subdomains, typical of natural variants.[7,22,45] We asked whether these enhancer alterations would result in changes in viral replication in mouse tissues upon inoculation of neonatal mice.

Balb/c mice that were less than 24 hours old were infected intra-peritoneally with approximately 1.2×10^6 pfu of the variants under study, using wild type A2 as a reference. The results of in-situ hybridization of mid-

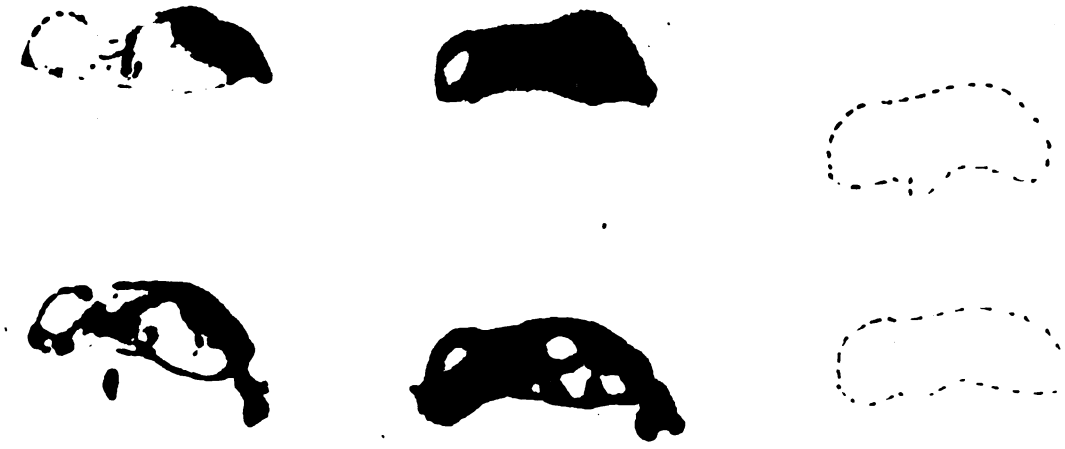
sagittal sections taken from 7 d.p.i. mice are shown in Fig. 3.

The A2 strain demonstrates the highest levels of polyoma sequences in the kidney tissue, salivary glands, and skin layers, followed by lesser levels in the liver, intestines, and lungs, with a notable absence of signal in the brain tissue and lumens of the stomach and bladder. When infections with enhancer variants are compared to that seen with A2, the following pattern of replication is seen. Viruses A2(A9), A2(SD-15), and A2(II-5) all exhibit patterns of replication in tissues identical to that of A2, with slight decreases seen in the total amount of sequences hybridizing in each section. In contrast, viruses A2(A8) and A2(NG-23) demonstrate a definite lack of replication when compared to the A2 pattern. This is a quantitative defect, for when these sections are exposed for longer periods, the same tissue pattern of replication is seen as was for the A2 strain, albeit at much lower intensities. We have observed these patterns of replication in a minumum of 8 mice for the A2(SD-15) and A2(II-5) viruses, up to 14 mice for the A2 and A2(A8) viruses. We conclude from these results that the viruses A2(A8) and A2(NG-23) have a defect in their ability to replicate in neonatal mouse tissues relative to A2, A2(A9), A2(SD-15), and A2(II-5) infected mice.

Figure 3. Replication of the A2(X) viruses in mouse tissues, utilizing in-situ hybridization.

Neonatal mice were infected with equivalent titers of the viruses indicated, and sacrificed 7 days post infection. Sections were then hybridized to polyoma specific probes as described in Methods. Mice infected with A2, A2(SD-15), A2(II-5), and A2(A9) have the highest levels of polyoma DNA present, while A2(A8) and A2(NG-23) have very low levels of viral DNA present. Note the complete lack of hybridization to non-infected sections.


A2(A-8)
A2(A-9)
A2(NG-23)
A2(SD-15)


A2(II-5)
A2
NON-INF.

To better quantitate the replication levels observed by in-situ hybridization, replication in specific organs was analyzed by extracting total DNA from the spleen, liver, lung, and kidney tissues of infected mice. The organ DNAs from three to six similarly infected mice were pooled, equivalent amounts of DNA were digested with BamHI, electrophoresed, transferred to nitrocellulose, and hybridized to a polyoma specific probe (Figure 4). Consistent with the results from in-situ hybridization, infections with wild type A2 strain generates the highest levels of polyoma DNA in the kidney, with liver, lung, and spleen harboring lesser levels. This pattern has been observed by others.[11] With viruses A2(SD-15), A2(A9), and A2(II-5) a very similar pattern is observed, with amounts of polyoma DNA nearly equivalent to those of A2 infected mice. As expected from the in-situ hybridization data, a very different pattern is observed with mutant viruses A2(A8) and A2(NG-23). Based upon densitometric scanning analysis A2(A8) and A2(NG-23) infections demonstrate a minimum of 30-fold decreases in the amount of viral DNA present in all four tissues analyzed. We conclude from both in-situ hybridization and tissue extracted DNA analysis that the enhancer rearrangements in strains A2(A8) and A2(NG-23) lead to a systemic defect in replication in neonatally infected mice.

Figure 4. Analysis of polyoma DNA sequences extracted from specific organs.

Neonatal mice were infected with the variant viruses being studied, sacrificed at 7 d.p.i., and the spleens, livers, lungs, and kidneys were dissected and pooled from equivalently infected mice. DNA was isolated as described in Methods, a BamHI partial digest of five micrograms of each sample was electrophoresed in a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a polyoma specific probe. The viruses are numbered as follows: 1=A2(A8), 2=A2(NG-23), 3=A2, 4=A2(A9), 5=A2(SD-15, and 6=A2(II-5).

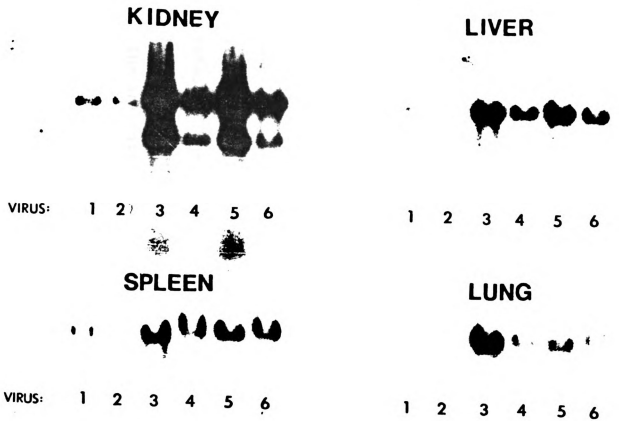


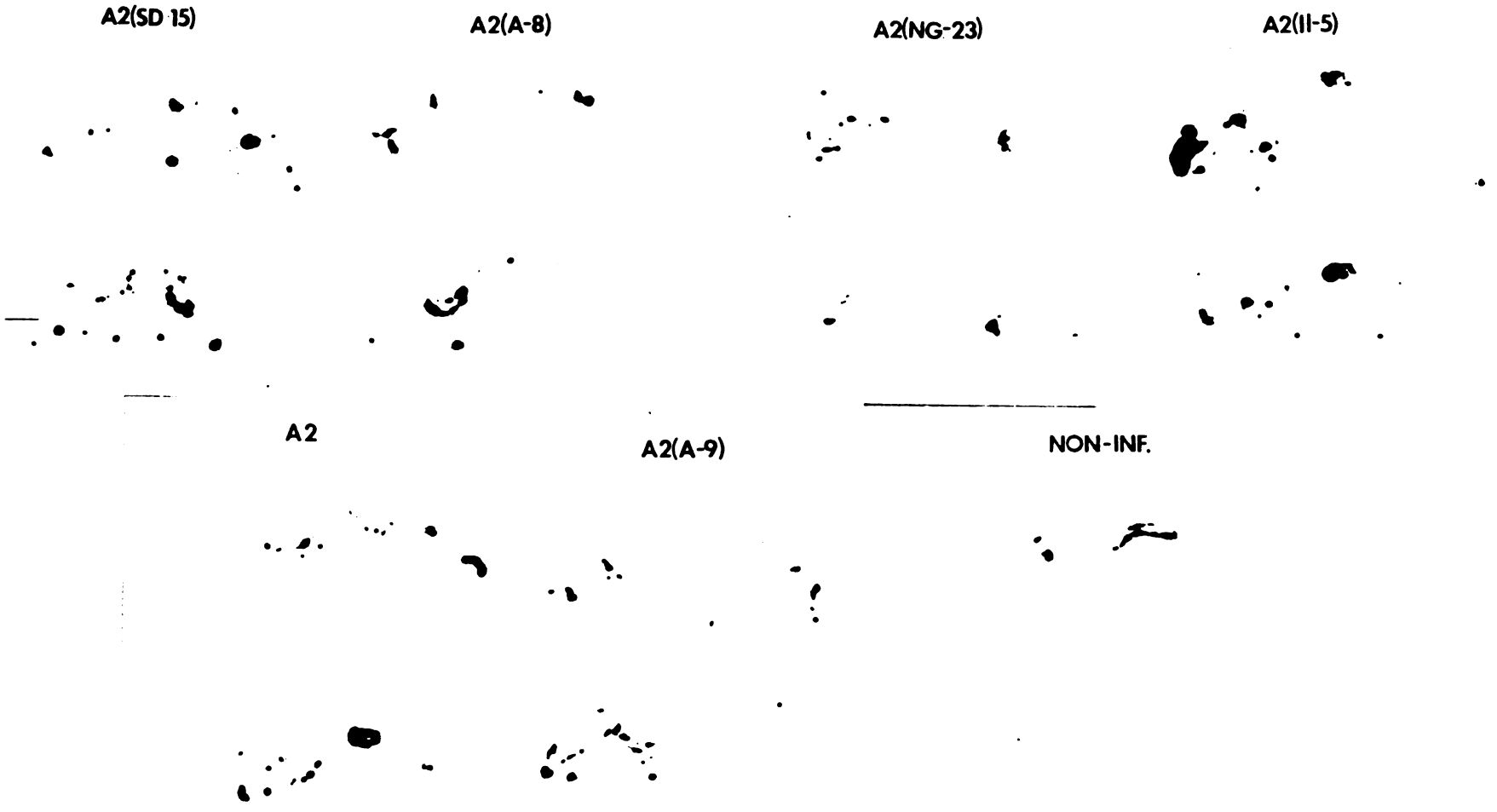
Figure 4. Analysis of polyoma DNA sequences extracted from specific organs.

Persistence studies in kidney tissue: Previous studies have demonstrated that in neonatally infected mice the kidney is a major target site for polyoma persistance at 4 w.p.i.[11,28] In-situ hybridization analyses of sagittal sections from a total of 38, 4 week old mice (6 per virus, 2 non-infected mice) proved more difficult than those of 7 day old mice due to an increase in non-specific binding of the probe coupled with an overall decreased level of viral DNA (see Methods). We did note, however, that in A2(A8), A2(NG-23), and possibly A2(A9) infections, the levels of viral DNA in the kidneys was reduced compared to that observed in A2 infections, or infections with the other A2(X) viruses (see Figure 5). While it is known that polyoma also persists in salivary tissue,[10,28] (as some of our sections demonstrate), we could not eliminate non-specific binding of our probe to these same regions in some non-infected control sections, making interpretation of hybridization in this region difficult.

The analysis of viral sequences extracted from kidneys of individual mice is shown in Figure 6. It demonstrates that in infections with A2, A2(SD-15), and A2(II-5) strains, viral sequences persist at nearly equivalent levels. In contrast, infection with strains A2(A8), A2(NG-23), and A2(A9) led to a 15 fold decrease in the amount of viral DNA sequences persisting in the kidney tissue of 4 week old mice.

Figure 5. Persistence of the A2(X) viruses in mouse tissues, utilizing in-situ hybridization.

Neonatal mice were infected with the designated viruses and sacrificed at 4 weeks post infection. Sections were hybridized to a polyoma specific probe as described in Methods. A2, A2(SD-15), and A2(II-5) consistently had viral DNA persisting in kidney tissue, unlike mice infected with A2(A8), A2(NG-23), and A2(A9). Polyoma specific probe is also observed to be hybridizing to salivary tissue in these sections. Note the non-specific binding of probe to the spinal column of one of the non-infected mice.


A2(SD 15)
A2(A-8)
A2(NG-23)
A2(II-5)
A2
A2(A-9)
NON-INF.

As expected from the low levels of primary replication observed at 7 d.p.i. for strains A2(A8) and A2(NG-23), very low levels of persistent replication were observed in kidney tissue at 4 w.p.i. In contrast, the A2(A9) strain, which was shown to replicate to near wild type levels at 7 d.p.i. (Figure 4) shows a defect in persistent replication at 4 w.p.i. This result suggests that high levels of viral accumulation at 7 d.p.i. is not the only requirement for high levels of polyoma DNA persistence up to 4 w.p.i. (see Discussion).

To analyze whether infections of the mouse select for new genetic variants, the viral DNA's recovered at 4 w.p.i. were compared to the the sequences of the original stocks, by restriction endonuclease analysis. MspI was chosen, as digestions with this enzyme gives rise to a fragment (#3) which contains the enhancer region, and easily reveals rearrangements around the origin (fragments 3 & 5) as well as deletions in other regions (Figure 7). The MspI analysis of the pooled 4 w.p.i. kidney DNA demonstrates that the size of fragment #3 from each strain, (as well as all other fragments) has been stable throughout the course of infection. This analysis will fail to reveal subtle DNA changes. However, the most common mutations of interest in the enhancer region usually are duplications and/or deletions.[1]

Figure 6. Analysis of polyoma DNA sequences extracted from kidneys of 4 w.p.i. mice.

Neonatal mice were infected with the indicated viruses, and sacrificed at 4 w.p.i. The DNA from the kidneys of individual mice was extracted, and 10 micrograms of each were digested with BamHI. The samples were electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a polyoma specific probe. The mice infected with the various viruses are indicated, and each lane represents DNA extracted from a single mouse.

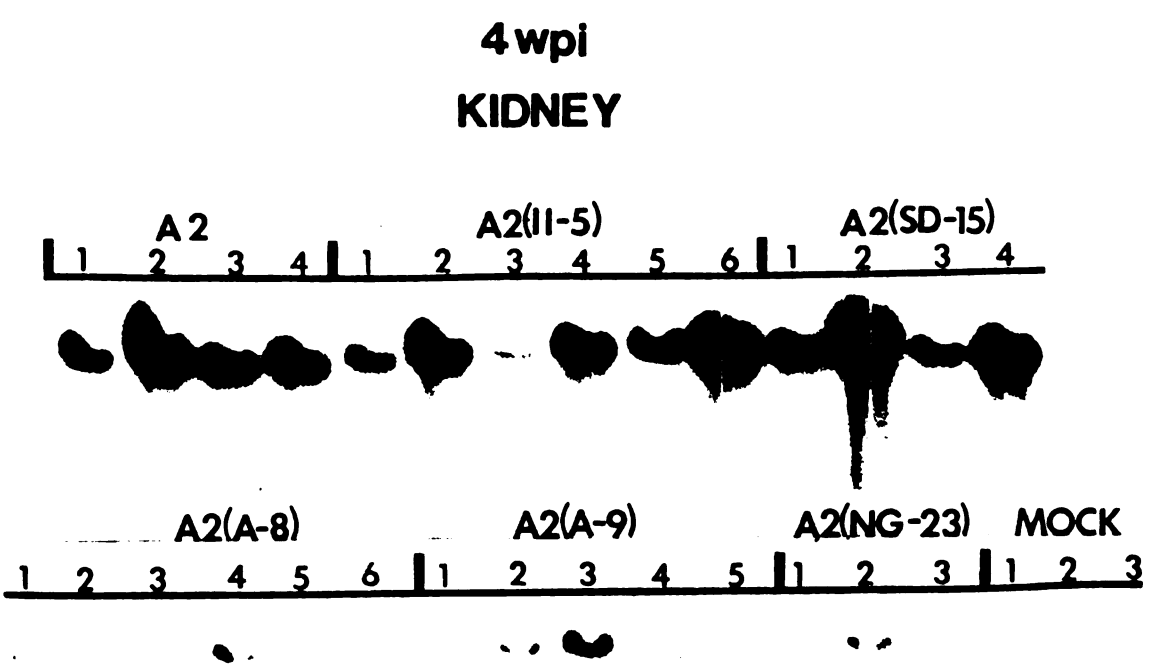


Figure 6. Analysis of polyoma DNA sequences from kidneys of 4 w.p.i. mice.

Finally, we have observed that mice which were infected with viruses which replicate to high levels at 7 d.p.i. (A2, A2(SD-15), A2(II-5), and A2(A9)) were more apt to display a previously described runting syndrome,[5] have inflammatory reactions of the eyes, and generally look sicker than mice infected with A2(A8), A2(NG-23), or non-infected mice. Possibly, the high levels of replication in the neonatal kidney tissue damages the tubules[25] allowing accumulation of toxic metabolites in the blood, causing a generalized runting phenomenon.

Discussion

We have begun to analyze what role the polyoma virus enhancer sequences have in modulating the tissue specific replication of the viral genome in the mouse. This question has been vigorously investigated in tissue culture systems, and the enhancer region has been shown to have a pivotal role in controlling host range.[1,14,20,22,27,29,42,45] Whether the findings in tissue culture are also important in infections of the mouse had not been specifically addressed. We have begun this analysis by utilizing natural virus isolates, which contain deletions and/or duplications of enhancer subdomains. These strains have been shown to replicate normally in mouse NIH-3T3 cells. For the purpose of facilitating this discussion, these alterations are diagrammed schematically in Figure 8.

Figure 7. MspI size analysis of viral DNA isolated from 4 w.p.i. kidney tissues.

The 4 w.p.i. kidney DNA's from equivalently infected mice were pooled, digested with MspI, electrophoresed in a 2% agarose gel, transferred to nitrocellulose, and hybridized to a polyoma specific probe. Only 5-10 micrograms of A2, A2(SD-15), and A2(II-5) pooled DNA were digested, while nearly 40 micrograms of A2(A8), A2(A9), and A2(NG-23) DNA had to be digested, (and exposed longer) to generate images of comparable intensity. The various MspI fragments are depicted (the enhancer rearrangements are within fragment #3). The original viruses used in the infections are as follows: A=A2, B=A2(SD-15), C=A2(II-5), D=A2(A8), E=A2(A9), and F=A2(NG-23)

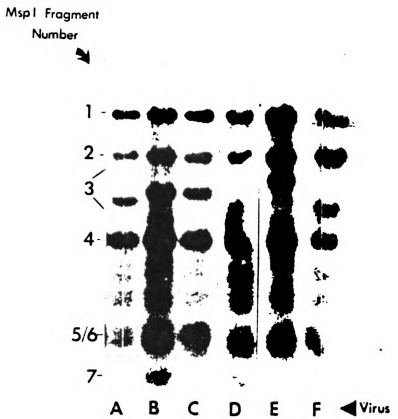


Figure 7. MspI size analysis of viral DNA isolated from 4 w.p.i. kidney tissues.

Our results demonstrate that enhancer rearrangements harbored by the viruses A2(A8) and A2(NG-23) lead to a significant decrease in replication compared to wild type A2 at early stages of the infection in neonates as measured at the peak of viremia (7 d.p.i) both by whole body in-situ hybridization, and extraction of organ DNA. Three types of differences are detected when comparing the sequence rearrangements in the enhancer regions of A2(A8) and A2(NG-23) to the A2 sequence (Figure 8) These suggest three possibile causes for the replication defect:

1. There are a number of single base pair additions, substitutions, and deletions in the A2(A8) and A2(NG-23) viral sequences beyond nucleotide 5265, on either side of the ori core of replication (denoted as Region A in Figure 8). The fact that other viruses, such as A2(SD-15) and A2(II-5), have these changes and are able to replicate to A2 levels at 7 d.p.i. suggests that these changes are not detrimental to viral replication.

2. The multiple duplications of sequences around nucleotides 5088-5141 (Region B, Figure 8) found in A2(A8) and A2(NG-23) may interfere with viral replication, possibly by increasing interactions with negatively acting factors present in mouse tissues. It has been demonstrated in tissue culture systems that the enhancer does interact with negatively acting factors.[4,47] However, the fact that

Figure 8. Physical map of the A2(X) viruses. The various duplications and deletions are represented, as previously described in Chapter 2.

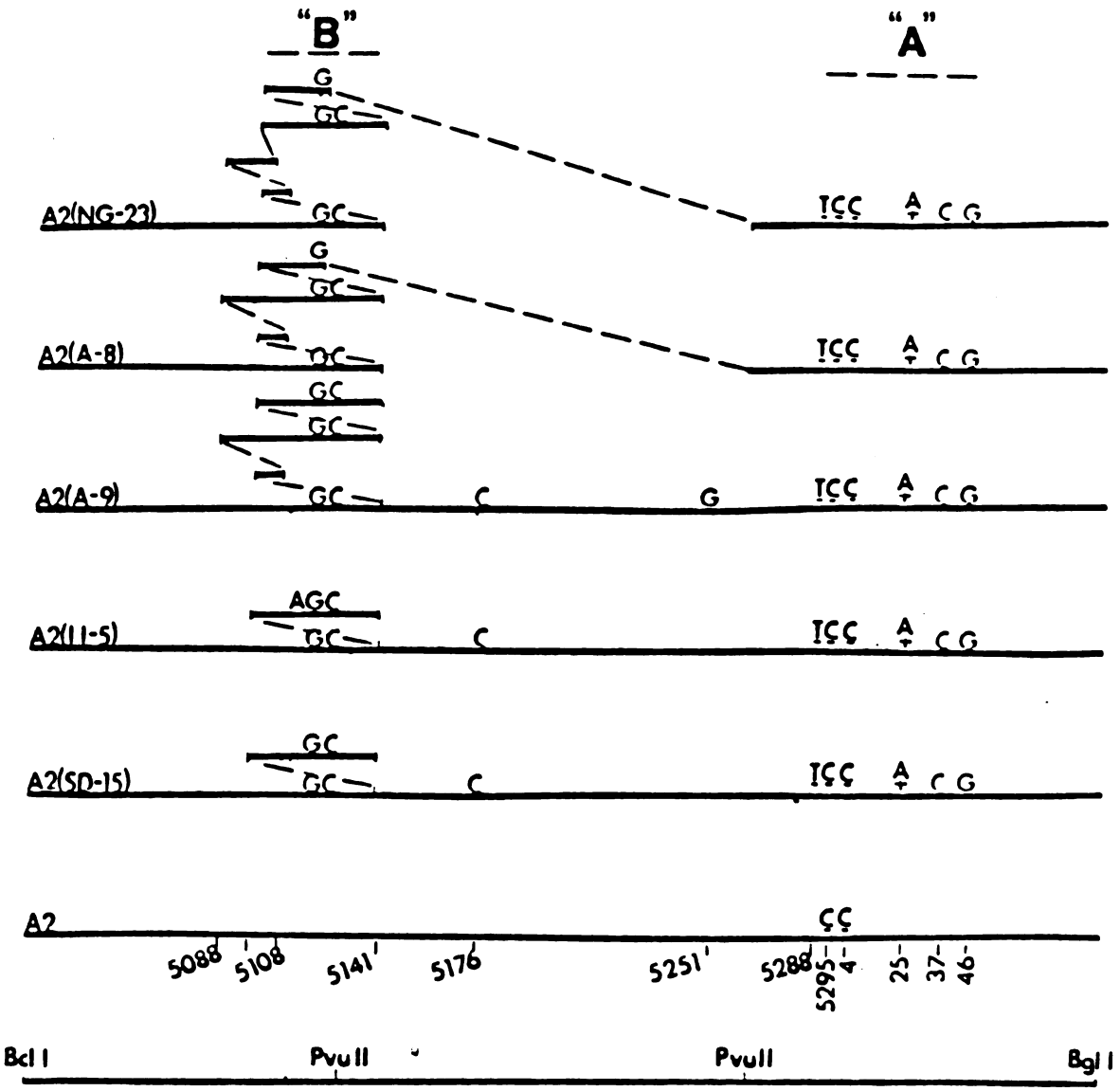


Figure 8. Physical map of the A2(X) viruses.

A2(A9) replicates to levels comparable to A2, with an enhancer region containing duplications nearly equivalent to those found in A2(A8), argues against that possibility.

3. Thus, by elimination, the 123 bp deletion present in both the A2(A8) and A2(NG-23) viruses appears to be responsible for the loss of replication in neonatal tissues. This suggests that a positively acting factor is interacting with sequences between nucleotides 5141-5265 of the polyoma genome in order to facilitate viral replication. This factor may be responsible for the high levels of replication demonstrated in neonatal tissues, in particular the kidney.

The polyoma enhancer region has been dissected into a number of subdomains in tissue culture systems,[19,20,30-32,44,46] as summarized in Fig. 9. Some of these domains are functionally redundant. The polyoma transcriptional enhancer domains are also replicational enhancer domains, thus blocks in replication are usually accompanied by blocks to transcription of viral early genes.[9,19,43,46] The nucleotides within 5141-5265, deleted in A2(NG-23) and A2(A8) define one of the two functionally redundant enhancer elements, (called B and C[46] or beta core and beta auxillary sequences[31]). These sequences have been shown to not be required for replication of polyoma in mouse NIH-3T3 cells (see Chapter 2), yet in actual mouse infections this

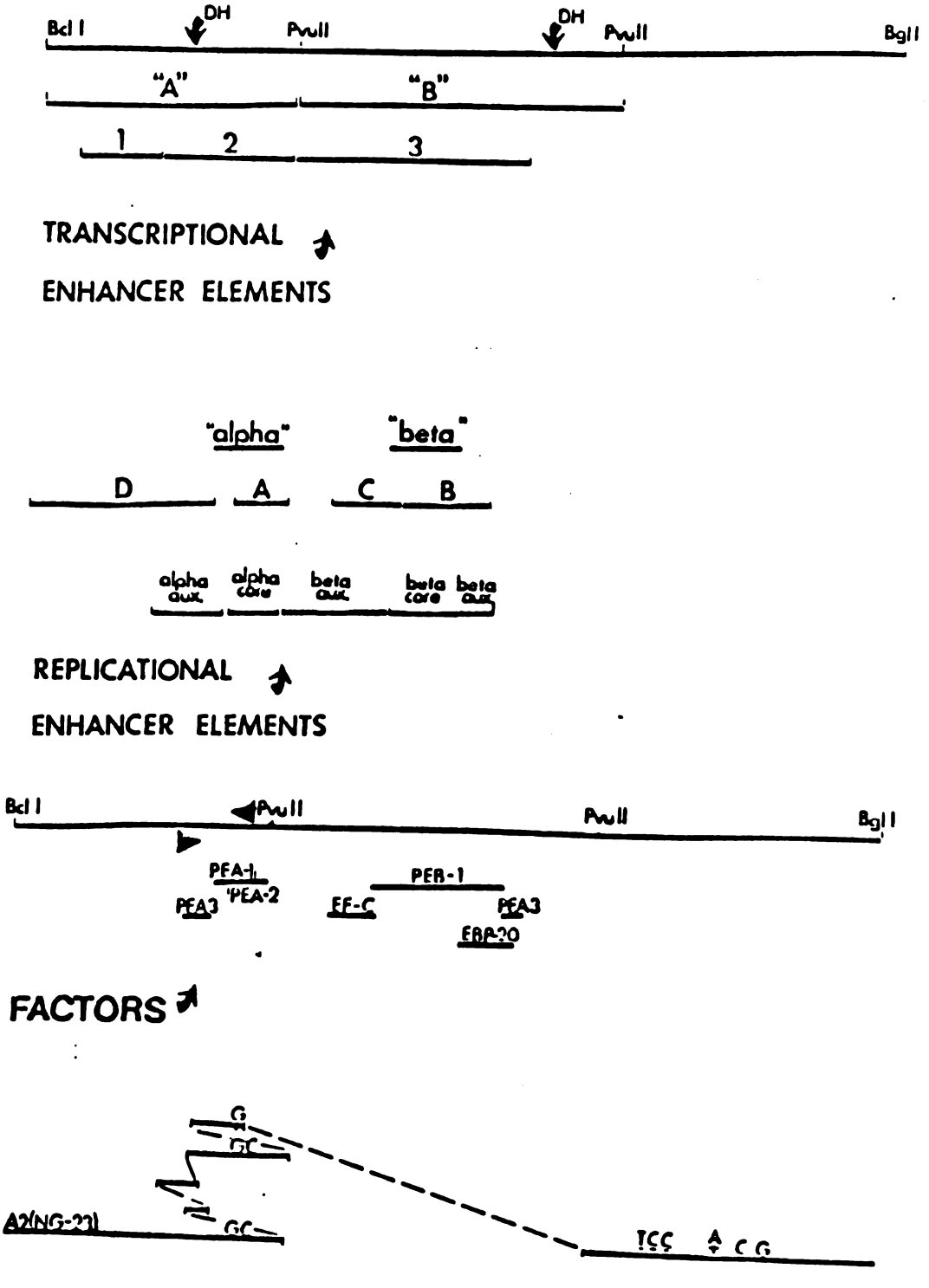


Figure 9. The transcriptional/replicational enhancer elements, and the factors which bind to this region.

region is crucial in allowing for high levels of viral replication.

The enhancer subdomains contained within this region have been demonstrated to bind to a number of cellular factors,[21,33-35] see Fig.9. The most intriguing of these factors in the context of our findings is EBP-20, a protein isolated from rat liver which was purified based upon its ability to bind to sequences 5211-5233 of the polyoma genome.[15,21] Possibly, a factor similar to EBP-20 is also present in neonatal mouse tissues, and controls the high level of viral replication shown by wild type viruses.

Interestingly, it has been demonstrated previously that a polyoma virus mutant with the B and C domains replaced by the LTR of the Moloney murine leukemia virus had lost the ability to replicate at normal sites in infections of neonate mice, and had simultaneously acquired a pancreatic tissue tropism.[36] Our results suggest that the pancreatic tropism was found because of the loss of the B and C domains, decreasing the levels of overall replication enough to allow visualization of the new tissue tropism induced by the LTR sequences.

The ability of the A2(X) viruses to persist in kidney tissue up to 4 w.p.i. was also investigated, and while the results are a bit more difficult to explain, they are intriguing nevertheless. The A2(SD-15), and A2(II-5) viruses persist in kidney tissue at a level equivalent to

that observed for wild type at 4 w.p.i., suggesting the sequence alterations in A2(SD15) and A2(II-5) are not deleterious to persistence. As expected, no persistence is observed with A2(A8) and A2(NG-23), most likely due to their inability to replicate to high levels early in the course of infection. Interestingly, the A2(A9) virus does not persist at 4 w.p.i. in kidney tissue, despite a normal early replication pattern. This suggests that persistence is not solely dependant on high level viral replication early in infections of mice, but that fulfillment of other criteria is also required.

Comparing the sequences of the viruses (see Figure 8) as we did earlier suggests two possible reasons for the persistence defect of A2(A9). One is that the additional duplications of sequences within nucleotides 5088-5141 (in relation to the single duplications found in the A2(SD-15) and A2(II-5) viruses) may allow for increased interactions with negatively acting factors in kidney tissue. This phenomenon could also contribute to the lack of persistence seen with the A2(A8) and A2(NG-23) viruses. The second possibility is that the single bp substitution at nt 5351 found in A2(A9) and not in A2, A2(SD-15), or A2(NG-23) may interfere with a positively acting factor required for viral persistance in kidney tissue. These two possibilites are being presently explored.

In conclusion, we have determined that the sequences of the polyoma genome between nucleotides 5141-5265 are required for high levels of viral replication, most likely by interactions with a positively acting factor present in neonatal mouse tissues. Secondly, persistence of polyoma DNA in kidney tissue at 4 w.p.i. is not only dependent on high levels of viral replication early in infection, but also on interactions with other positive or negatively acting factors.

References

1. Amati, P. (1985). Polyoma regulatory region: A potential probe for mouse cell differentiation. Cell 43: 561-562.

2. Berebbi, M., Dandolo, L., Hassoun, J., Bernard, A.M., Blangy, D. (1988). Specific tissue targeting of polyoma virus oncogenicity in athymic nude mice. Oncogene 2: 149-156.

3. Buffet, R.F., Levinthal, J.D. (1962). Polyoma virus infection in mice. Pathogenesis Arch. Pathol. (Chicago) 74: 513-526.

4. Cremisi, C., Babinet, C. (1986). Negative regulation of early polyomavirus expression in mouse embryonal carcinoma cells. J. Virology 59:761-763.

5. Dawe, C.J. (1960). Cell sensitivity and specificity of response to polyoma virus. J. National Cancer Inst. Monograph No. 4. Symposium on Phenomenon of the Tumor Viruses. 67-92.

6. Dawe, C.J., Freund, R., Mandel, G., Ballmer-Hofer, K., Talmadge, D.A., Benjamin, T.L. (1987). Variations in polyoma virus genotype in relation to tumor induction in mice. Amer. J. Path. 127:243-261.

7. DeSimone, V., Amati, P. (1987). Replicative cis-advantage of polyomavirus regulatory region mutants in different murine cell lines. J. Virology 61:1615-1620.

8. deVilliers, J., Schaffner, W. (1981) A small segment of polyoma virus DNA enhances the expression of a cloned beta-globin gene over a distance of 1400 base pairs. Nucleic Acids Research 9:6251-6264.

9. deVilliers, J., Schaffner, W., Tyndall, C., Lupton, S., Kamen, R. (1984). Polyoma virus DNA replication requires an enhancer. Nature (London) 312:242-246.

10. Dubensky, T.W., Murphy, F.A., Villarreal, L.P. (1984). Detection of DNA and RNA virus genomes in organ systems of whole mice: Patterns of mouse organ infection by polyomavirus. J. Virology 50:779-783.

11. Dubensky, T.W., Villarreal, L.P. (1984). The primary site of replication alters the eventual site of persistant infection by polyomavirus in mice. J. Virology 50:541-546.

12. Freund, R., Dawe, C.J., Benjamin, T.L. (1988). Duplication of noncoding sequences in polyomavirus specifically augments the development of thymic tumors in mice. J. Virology 62:3896-3899.

13. Freund, R., Dawe, C.J., Benjamin, T.L. (1988). The middle T proteins of high and low tumor strains of polyomavirus function equivalently in tumor induction. Virology 167:657-659.

14. Fujimura, F.K., Silbert, P.E., Eckhart, W., Linney, E. (1981). Polyoma virus infection of retinoic acid-induced differentiated teratocarcinoma cells. J. Virology 39: 306-312.

15. Graves, B.J., Johnson, P.F., McKnight, S.L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell 44:565-576.

16. Gross, L., (1953). A filterable agent, recovered from Ak leukemic extracts, causing salivary gland carcinomas in C3H mice. Proc. Soc. Exp. Biol. Med. 83: 414-421.

17. Haase, A.T., Brahic, M., Stowring, L., Blum, H. (1984). Detection of viral nucleic acids by in situ hybridization, in "Methods in Virology" 7:189-226.

18. Harper, J.S., Dawe, C.J., Trapp, B.D., McKeever, P.E., Collins, M., Woyclechowska, J.L., Madden, D.L., Sever, J.L. (1983). Paralysis in nude mice caused by polyomavirus-induced vertebral tumors. Polyomaviruses and Human Neurological Diseases 359-367.

19. Hassell, J.A., Muller, W.J., Mueller, C.R. (1984). The dual role of the polyomavirus enhancer in transcription and DNA replication. Cancer Cells 4: DNA tumor viruses, 561-569.

20. Herbomel, P., Bourachot, B., Yaniv, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39:653-662.

21. Johnson, P.F., Landschulz, W.H., Graves, B.J., McKnight, S.L. (1987). Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes and Development 1:133-146.

22. Katinka, M., Vasseur, M., Montreau, N., Yaniv, M., Blangy, D. (1981). Polyoma DNA sequences involved in control of viral gene expression in murine embryonal carcinoma cells. Nature (London) 290:720-722.

23. Kovesdi, I., Satake, M., Furukawa, K., Reichel, R., Ito, Y., Nevins, J.R. (1987). A factor discriminating between the wild-type and a mutant polyomavirus enhancer. Nature (London) 328:87-89.

24. Kryszke, M.H., Piette, J., Yaniv, M. (1987). Induction of a factor that binds to the polyoma virus A enhancer on differentiation of embryonal carcinoma cells. Nature (London) 328:254-256.

25. Levinthal, J.D., Jakobovits, M., Eaton, M.D. (1962). Polyoma disease and tumors in mice: The distribution of viral antigen detected by immunofluorescence. Virology 16:314-319.

26. Luthman, H., Nilsson, M.G., Magnusson, G. (1982). Non-contiguous segments of the polyoma genome required in cis for DNA replication. J. Mol. Biol. 161:533-550.

27. Maione, R., Passananti, C., De Simone, V., Delli-Bovi, P., Augusti-Tocco, G., Amati, P. (1985). Selection of mouse neuroblastoma cell-specific polyoma virus mutants with stage differentiative advantages of replication. EMBO 4: 3215-3221.

28. McCance, D.J. (1981). Growth and persistence of polyoma early region deletion mutants in mice. J. Virology 39:958-962.

29. Melin, F., Pinon, H., Reiss, C., Kress, C., Montreau, N., Blangy, D. (1985) Common features of polyomavirus mutants selected on PCC4 embryonal carcinoma cells. EMBO 4: 1799-1803.

30. Mueller, C.R., Mes-Masson, A.M., Bouvier, M., Hassell, J.A. (1984). Location of sequences in polyomavirus DNA that are required for early gene expression in vivo and in vitro. Mol. Cell. Biol. 4:2594-2609.

31. Muller, W.J., Dufort, D., Hassell, J. (1988). Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. Mol. Cell. Biol. 8:5000-5015.

32. Muller, W.J., Mueller, C.R., Mes, A.M., Hassell, J.A. (1983). Polyomavirus origin for DNA replication comprises multiple genetic elements. J. Virology 47: 586-599.

33. Piette, J., Kryszke, M.H., Yaniv, M. (1985). Specific interaction of cellular factors with the B enhancer of polyoma virus. EMBO 4:2675-2685.

34. Piette, J., Yaniv, M. (1986). Molecular analysis of the interaction between an enhancer binding factor and its DNA target. Nucl. Acids Res. 14:9595-9611.

35. Piette, J., Yaniv, M. (1987). Two different factors bind to the alpha domain of the polyoma virus enhancer, one of which also interacts with the SV40 and c-fos enhancers. EMBO 6:1331-1337.

36. Rochford, R., Campbell, B.A., Villarreal, L.P. (1987). A pancreas specificity results from the combination of polyomavirus and Moloney murine leukemia virus enhancer. Proc. Natl. Acad. Sci. USA 84: 449-453.

37. Rowe, W.P. (1961). The epidemiology of mouse polyoma virus infection. Bacteriol. Revs. 25:18-31.

38. Rowe, W.P., Hartley, J.W., Estes, J.D., Huebner, R.J. (1959). Studies of mouse polyoma virus infection. I. Procedures for quantitation and detection of virus. J. Exper. Med. 109: 379-391.

39. Salzman, N.P. ed. (1986). The papovaviridiae. Volume I: The Polyomaviruses. Plenum Press, New York and London.

40. Southern, P.J., Blount, P., Oldstone, M.B.A. (1984). Analysis of persistent virus infections by in situ hybridization to whole-mouse sections. Nature (London) 312:555-558.

41. Stewart, S.E., Eddy, B.E., Borgese, N. (1958). Neoplasms in mice inoculated with a tumor agent carried in tissue culture. Journal of the National Cancer Inst. 20:1223-1243.

42. Tanaka, K., Chowdhury, K., Chang, K.S.S., Israel, M., Ito,Y. (1982). Isolation and characterization of polyoma virus mutants which grow in murine embryonal carcinoma and trophoblast cells. EMBO 1:1521-1527.

43. Tang. W.J., Berger, S.L., Triezenberg, S.J., Folk, W.R. (1987). Nucleotides in the polyomavirus enhancer that control viral transcription and DNA replication. Mol. Cell. Biol. 7:1681-1690.

44. Tyndall, C., LaMantia, G., Thacker, C.M., Favaloro, J., Kamen, R. (1981). A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nuc. Acids Res. 9:6231-6250.

45. Vasseur, M., Kress, C., Montreau, N., Blangy, D. (1980). Isolation and characterization of polyoma virus mutants able to develop in embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 77: 1068-1072.

46. Veldman, G.M., Lupton, S. Kamen, R. (1985). Polyomavirus enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. 5:649-658.

47. Wasylyk, B., Imler, J.L., Chatton, B., Schatz, C., Wasylyk, C. (1988). Negative and positive factors determine the activity of the polyoma virus enhancer alpha domain in undifferentiated and differentiated cell types. Proc. Natl,. Acad. Sci. USA 85:7952-7956.

Summary and Conclusions

The role of the polyoma enhancer region in directing replication of the viral genome was investigated. The experiments described in Chapter 2 utilized tissue culture systems and recombinant viruses to define a subdomain of the polyoma enhancer as either essential for allowing viral replication in PCC4 embryonal carcinoma cells, or expendable in mouse fibroblasts. The presence of this domain had been thought to be required of all viable viruses since none had ever been isolated which lacked this domain. The results presented here suggest that further studies should be done to describe the exact nature of the functional redundancy exhibited by the viruses A2(A8) and A2(NG-23). Possibly interactions with new host proteins, and/or modified interactions with previously described factors is the cause of the effect. Thus, a firmer understanding of the processes of gene expression and DNA replication may be gained by such studies.

The studies in Chapter 3 demonstrated that viruses which could replicate to wild type levels in tissue culture systems did not always behave accordingly when actual mice were infected. This demonstrates one of the few shortcomings of tissue culture systems, that the results obtained in tissue culture may not accurately reflect the

true interactions at the host level. Nevertheless, the results demonstrated that modifications of the enhancer region alone, (without changes in viral capsid structure, or structural proteins) could have a profound effect on the ability of viruses to grow in normally permissive host tissues. The regions of the enhancer defined as crucial (in Chapter 3) to allow for high-level viral replication indicates that there may be specific factors in the mouse tissues interacting with this particular enhancer domain. Protein extracts from mouse tissues may contain specific enhancer binding proteins specific for this region. These factors may be central to gene expression in general, since loss of this enhancer domain abolished viral replication in a number of mouse tissues.

Finally, the results presented here suggest that the pathogenicity of other viruses may be altered simply by altering their respective enhancer regions, preventing viral gene expression in host tissues. This sort of approach, along with the more traditional ways of attenuating viruses, may allow for better vaccine production.

Appendix

Cover Sheet

The following paper, in which I appear as an author, is included here because I was responsible for a large portion of the data. This includes all data referred to in Figures 3-6, and Experiments 2b-5c listed on Table I.

Prepared for Mol. Cell. Biol.

Appendix

LOW PROBABILITY OF DOUBLE INTEGRATION IN TRANSFORMATION OF NONPERMISSIVE CELLS BY POLYOMA VIRUS

Saw Yin Oh[+], Andrea Amalfitano, Karen Friderici[++], Ming Chu Chen, and Michele M. Fluck[*]

Department of Microbiology, Interdepartmental Program in Molecular Biology
Michigan State University
East Lansing, MI 48824-1101
(517) 353-5014

Running title: Integration of unselected polyoma genomes

This is journal article no. from the Michigan Agricultural Experiment Station.

Present Address: [+]Department of Pharmacology, Fluiders University, Bedford Park, South Australia 5042
[++]Department of Pathology, Michigan State University, East Lansing, Michigan 48824

Abstract

F-111 rat and BHK hamster cells were infected with mixtures of wild type polyoma virus and transformation-defective deletion mutants. The viral integration patterns of resulting transformants were determined. In the majority of the transformants the unselected deletion mutant was not present, even when the unselected parent was used in large excess over the wild type in the infection mix. These results were obtained for transformants derived with a variety of parental viral strains. Overall, the unselected parent was recovered in only 11% of the transformants analyzed (i.e. in 14 of 142). When two transformation competent strains were used to infect rat cells, a low frequency of double parental integration was also observed. It was shown that both parents in the infection mix are infecting the same cells and that both replicate and persist in the infected cells up to the time when transformants arise. These results suggest that the probability for two independent integration events per transformant is low, in contradiction to previous results on the analysis of the number of integration sites determined by hybridization of restriction endonuclease fragments separated by electrophoresis. We present a reinterpretation of these data in agreement with the present data.

Introduction

The pattern of viral integration concomitant with neoplastic transformation by papovaviruses polyoma and SV-40 has been extensively studied. Analysis of cellular DNA from transformants by electrophoretic separation of restriction endonuclease fragments has shown that the viral genome is apparently inserted at multiple sites in the host chromosome, where it is arranged in a head-to-tail manner (4,5). The variation in patterns from transformant to transformant suggests a great diversity in chromosomal integration sites. A review of the published literature concerning the number of integration sites of the polyoma viral genome in rat transformants suggests multiple sites in 80% of the cases reported--the number of sites per transformant averaging over three (1,4,8,15,20,29). In experiments using gels with more resolving power, this number appears to be even higher (i.e., eight to ten) (16 and K. Friderici and M. Fluck, unpublished). Thus, it has generally been assumed that the number of chromosomal sites available for integration is very high, if not unlimited. The simplest model would assume 1) that each of the multiple integration events in any transformant represents a random interaction between available sites on the host chromosome and available members from the pool of integratable viral genomes and 2) that each event happens independently from the events occurring at other sites.

Assuming that all genomes present in the parental infection mix have equal probability to become integrated, we would expect the integrated genomes to be a faithful representation of all the viral genomes present in the input for the infection. To test this prediction, we have carried out mixed infections of nonpermissive rat and hamster cells with wild type and

transformation defective deletion mutants, using various multiplicities and ratios of the two parents. We isolated transformants from such infections and studied the viral integration patterns of the two parental genomes. It was expected that, in addition to the selected transforming wild type parent, the transformation-defective mutant would be recovered in the transformants at a frequency and a dosage which reflect the ratio of the two genomes in the input of infection. The results of our test are described in the present report. They failed to support the prediction from the model, and consequently, generated hypotheses for an alternate model.

Materials and Methods

Cells and Viruses. Fischer rat F-111 (10), and hamster BHK cells (28) were grown in Dulbecco's modified Eagle's medium supplement with 10% newborn calf serum.

Wild type strains A2 and A3 are widely used strains which have been sequenced (16,24). The pseudo-wild-type strain NG59RA (RA) was obtained by marker rescue (11) of the middle T-antigen defect of hr-t mutant NG59 (3). An unexpected large T-antigen defect of this strain has been described elsewhere (18). Differences between RA and A2 in the enhancer region have been noted previously (24). RA and A2 also differ in plaque morphology: RA is a small plaque virus with a heat stable hemagglutination pattern (6), while A2 is a large plaque virus. Transformation-defective hr-t mutants B2, 30'B, and 3A3 were derived from the Pasadena small plaque strain by mutagenesis with ICR-191 (27). All hr-t mutants used contain rearranged enhancer regions (11 and K. Higgins, K. Friderici and M. M. Fluck, unpublished) as well as a deletion in the middle T-antigen gene located in HpaII/MspI fragment 4 (3,11,16). This deletion is also easily detectable in PvuII digestions. Transformation defective mutant 18-5 was constructed from wild type A2 and was a gift of M. Fried (20). It too contains a deletion in MspI/HpaII fragment 4. Wild type RA-B2Ori was constructed by ligating the small BglI-BamHI fragment encompassing the enhancer-origin region of hr-t mutant B2 to the large genomic BglI-BamHI fragment of pseudo wild type RA. Mutant d145, derived from wild type A3 (2), has a deletion of 66 nucleotides in the overlapping large T-antigen/middle T-antigen reading frames. The deletion can be visualized in digestion with Sst I. This mutant is reported to have no transformation nor growth defects (2).

Infections. F-111 or BHK cells were seeded at a density between 10^5 and 1.5 x 10^6 cells per 60 mm plate (as described in the text and Table 1) and infected at the multiplicities and ratios of mutant to wild type virus indicated in the text and Table 1. In most instances, the relative ratio of the two parental genomes in the infection mix, established from the titer of the two parental viral stocks, was confirmed by hybridization analysis of the viral sequences in the input. Cells were fed with DMEM supplemented with 5% newborn calf serum and became confluent within one to eight generations postinfection (see Table 1). In experiment 4b and 5b, each infected 60 mm dish was passed to ten 100 mm dishes (leading to an approximate cell confluency of 1.5 and 3%, respectively) and fed with DMEM supplemented with 10% serum until confluent. In most cases, transformants from infections carried out under the various conditions described were isolated as foci overgrowing a cell monolayer. In some cases, transformants were isolated directly (or were recloned) by growth in agar.

DNA Analysis. Total cellular DNA was isolated from infected or transformed cells as described (12,21). For gel electrophoresis, each sample contained 10-20 µg DNA digested with the restriction enzymes indicated in the text and figure legends. Endonucleases were chosen for their ability to generate fragments with which wild type and deletion mutant sequences can be distinguished. MspI/HpaII or PvuII were chosen for all infections with hr-t mutants (exp. 1-8 and 10, Table 1), and SstI to distinguish between A3 and dl45 (experiment 9, Table 1). After transfer to nitrocellulose (21,26), the blots were hybridized to polyoma nick translated probes (1-2 x 10^8 cpm/µg). Hybridization was at 65°C for 3 days in 2XSSC/1X Denhardt's (0.1 ml/cm^2 using 1 x 10^6 cpm of the labeled probe per

ml of hybridization solution). In many of the hybridizations a probe consisting of HpaII fragment 4 and designated pPyH4 was utilized. Since the hr-t deletions are contained within the fragment 4 generated by restriction endonuclease HpaII or isoschizomer MspI, this probe allows the identification of wild type or mutant sequences spanning this area of the genome (12).

RESULTS

An assay for the integration of unselected viral genomes. To analyze the integration of viral genomes unselected by the transformation process we established transformants from infected nonpermissive cells, with mixtures of transforming and nontransforming viral strains containing deletions in their genomes (see Materials and Methods). Such transformants are expected to contain an integrated wild type genome selected in the transformation process. The integration of the unselected transformation-defective deletion genome present in the infection mix was detected by means of restriction endonucleases which distinguish between the wild type and the deleted parental genomes (see Materials and Methods). A total of 120 transformants were analyzed; these were derived from ten different infections involving four different wild type viruses and four deletion mutant viruses. These results are summarized in Table 1.

Normal overall integration patterns in transformants derived from mixed infections. The viral integration pattern of transformants resulting from the mixed infections were analyzed by restriction endonuclease digestion, electrophoresis, Southern blotting and hybridization to viral probes. The patterns obtained for transformants derived from co-infections of Fischer rat F-111 cells with wild-type strain RA and transformation defective hr-t deletion mutant B2 are shown Fig. 1. In the experiment shown, the two parental genomes were used at equal multiplicity of infection. In 8 out of 10 transformants presented in Fig. 1 (experiment 1, Table 1), a typical integration pattern is observed: 1) first and most important, multiple apparent sites of integration are present, as evidenced by multiple bands in digestions with restriction endonuclease BglII which does

Table 1. Integration of an unselected viral genome.[a]

[a] Summary of the transformants analyzed in this report. Rat or hamster cells were co-infected as described in Materials and Methods by the deletion mutant and transforming parent indicated. The multiplicities of infection for each parent are indicated.

[b] The deletion mutants described in Materials and Methods which can be distinguished from the wild type by the presence of deletions in their genomes. B2, 30'b, and 3A3 are transformation defective "hr-t" mutants, which are not selected in the transformation process; these are easily distinguished from wild type sequences in digestion with MspI/HpaII or PvuII. Mutant d145, used in experiment 9, is a transformation competent strain, which can also be distinguished from wild type A3 because of a 66 bp deletion in its genome. The deletion is easily visualized in digestions with SstI.

[c] WT refers to the source of the transforming parent. For a description of these strains, see Materials and Methods. In experiment 9, either parent (A3 or d145, or both) can contribute to the transformation phenotype.

[d] The ratio of the multiplicities of nontransforming to transforming mutant which were used to obtain the transformants. In most cases, the ratio of the two parental genomes in the infection mix, established by relying on the titles of the parental viral stocks, was confirmed by hybridization analysis of the infection mix.

[e] The number of cell divisions allowed postinfection until infected cells formed a confluent monolayer. This number depends on the cell density at the time of infection (see Materials and Methods). Cells were set in 60 mm dishes at: i) 2×10^5; ii) 5×10^5; iii) 1×10^6 cells per dish. In ', infected cells were passed to 10 plates within 24 hrs postinfection. In A, infected cells were passed to agar immediately postinfection.

[f] Summary of the number of transformants containing the unselected viral genome over the number of transformants analyzed as determined by Southern blotting analysis and illustrated in Fig. 3.

[g] The ratio in percent of transformation frequency in the mixed infection over transformation frequency in the infection with the transforming parent alone. NA no applicable. ND not done.

Table 1. Integration of an Unselected Viral Genome[a]

Exp.	Mutant[b]	"WT"[c]	MOI[d]	Host	# of cell divisions[e]	Mutant Integrations[f]	% WT Transformation[g]
1	B2	RA	5:5	F-111	2-3[1]	0/10	ND
2a	B2	RA	1:1	F-111	2-3[1]	0/4	400
2b			5:1			0/3	1700
3	B2	RA	20:0.1	F-111	8[1,']	1/14	200
4a	B2	RA	20:1	F-111	1[11]	2/6	ND
4b					4-5[1,']	1/6	ND
5a	B2	RA	10:10	F-111	0[111]	2/12	ND
5b					6-7[111,']	4/12	ND
5c			2:2		6-7[111]	1/6	
6	B2	RA-B2Ori	20:4	F-111	2-3[1]	0/12	150
7	30'b	A2	5:1	F-111	2-3[1]	1/5	30
8	3A3	A2	20:1	F-111	2-3[1]	0/5	18
9	d145	A3	10:10	F-111	3, 0[4]	both 2/12, 4/22	NA
						d145 2/12, 10/22	
						A3 8/12, 8/22	
10	B2	RA	5:5	BHK		2-3[1] 1/13	ND
		Total				16/142	

not cut the viral genome (Fig. 1A) (for example, the transformant analyzed in lane 2 shows seven bands apparently representative of integration sites, including an unresolved band at limit entry which appears to represent more than a single band); 2) free viral genomes are evidenced by a band of identical size in all samples co-migrating with supercoiled viral DNA in the same BglII digests (Fig. 1A), and 3) the viral genome is integrated as a head-to-tail tandem, as revealed by a genome size 5.3 kbp band in digestions with EcoRI which cuts the polyoma genome at a single site (Fig. 1B). Only two transformants in 10 show a single site of integration containing an incomplete viral genome and no free viral DNA in this particular set (lanes 1 and 8 Fig. 1A and 1B). Another 63 rat transformants derived from other mixed infections with RA and B2 were analyzed and found to have "normal" integration patterns (experiments 2, 3, 4, 5, Table 1) as well as 12 transformants derived from infections with B2 and wild type RA-B2Ori (exp. 6). This is also the case in transformants derived from co-infections of hr-t mutants 30'b or 3A3 with wild type A2 (10 cases analyzed) (experiments 7, 8, Table 1). Apparently, most of the Fischer rat transformants analyzed (95%) contain evidence for multiple integration sites as judged by analysis with a restriction endonuclease which does not cut the viral genome. Note that the number of flanking bands in digests with EcoRI is less than expected (compare lane 2 in Fig. 1A and 1B). We will return to this apparent paradox in the discussion.

Low frequency of integration of the unselected parent. The transformants described in Fig. 1 were further analysed for the presence of the two parental genomes. Each parent can be easily distinguished by the presence of a normal or a deleted restriction nuclease fragment. Fig. 2

Fig. 1. Integration patterns in F-111 cells.

Rat F-111 cells (2 x 10^5 cells per 60 mm dish) were infected at a multiplicity of five plaque-forming units each of transforming strain RA and transformation defective deletion mutant B2 (experiment 1, Table 1). Independent transformed foci were selected and cloned by anchorage independence in soft agar. 10 to 20 μg of high molecular weight DNA isolated from these clones was digested with BglII (A) or EcoRI (B) and fractionated on 0.6% agarose gels. Hybridizations were as described in Materials and Methods using 10^6 cpm/ml of a probe (pPy-1) representing the whole genome. M shows the position of marker viral DNA digested with EcoRI and the arrow shows the position of supercoiled form I unintegrated viral DNA. Lane 1-10 contain DNA from individual foci.

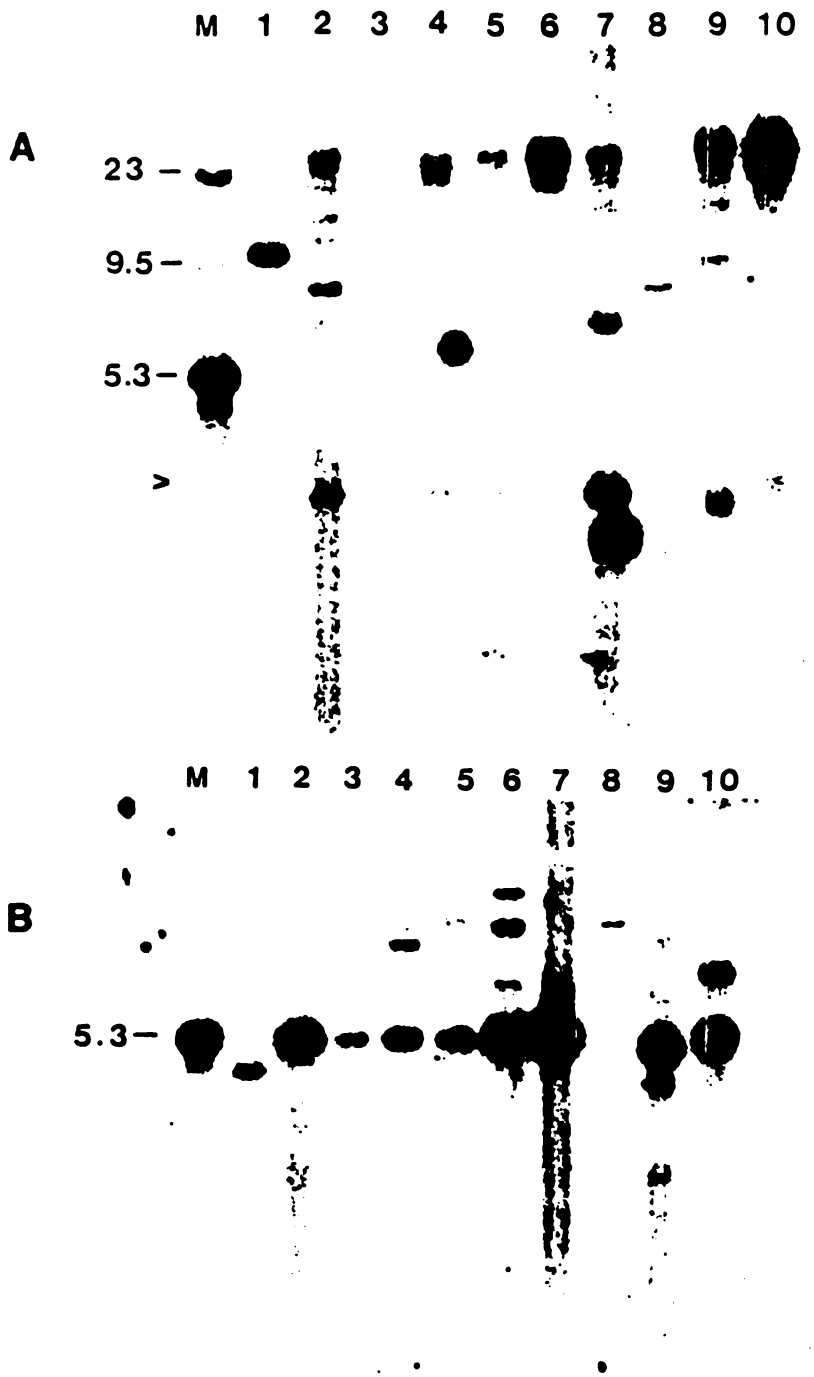


Figure 1. Integration patterns in F-111 cells.

shows the results for 10 Fischer rat transformants from Table 1, experiment 1, digested with restriction enzyme MspI. The probe used in these experiments is the MspI/HpaII fragment 4 in which the B2 deletion (nucleotides 491-730) is located (6). All transformants contain a normal size fragment 4 corresponding to the presence of the wild type parent. This is expected since fragment 4 is in the coding region for middle T-antigen which is required for transformation. In contrast, none of 10 F-111 transformants contain the B2 parent. This is surprising for 8 of 10 of these transformants which apparently have evidence for multiple integration events. Similar underrepresentation of the B2 mutants was observed in other experiments in which the RA and B2 parental genomes were used in equal ratios at varying total multiplicity of infection. The B2 parent was recovered in none of four transformants analyzed in experiment 2a, in two of 12, four of 12 and one of six in experiment 5 a,b,c, respectively.

To increase the probability of mutant recovery, the ratio of the unselected to the selected parent was varied in the infection mix in favor of the unselected parent. In the most extreme case, the experiments were repeated using a 200-fold excess of the unselected parent (i.e., multiplicities of 20 of the unselected parent and 0.1 of the selected transforming parent [experiment 3]). In such conditions, one should be able to achieve an effective ratio of mutant to wild type of 20 to 1 per transformable cell, i.e., in each cell which receives a wild type (experiment 3, Table 1). The high ratio of unselected to selected parent was confirmed by hybridization of the HpaII digested viral DNA extracted either from the infecting mix or from infected cells at 2 hours post infection. In these, the mutant shows a dense band while the wild type sequences are below the

Fig. 2. Low frequency of integration of the unselected parent among the integrated viral sequences in rat transformants.

The rat F-111 transformants described in Fig. 1 were further analyzed for the presence of the unselected parental genome. 10 to 20 μg of DNA was digested with MspI and fractionated on 2% agarose gels. Blotting and hybridizations were as described in Materials and Methods. The probe used is pPyH4, representing the MspI fragment 4 in which the hr-t deletion is located.

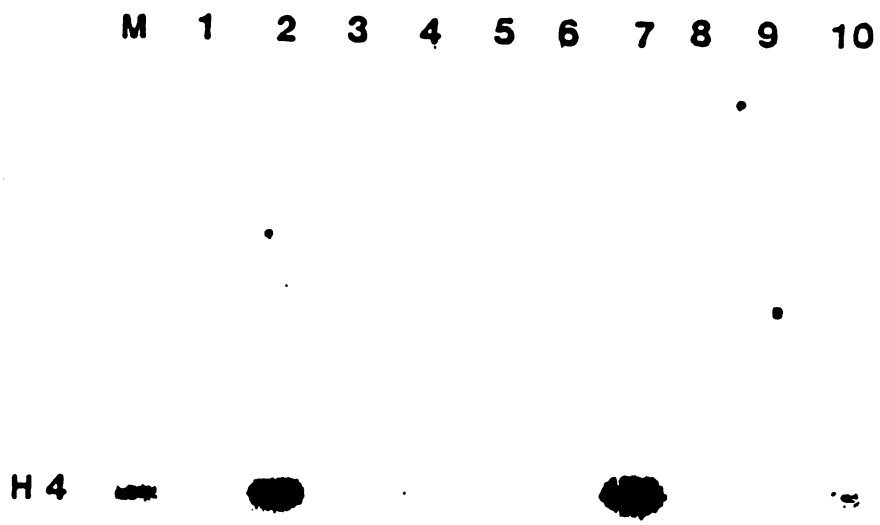


Figure 2. Low frequency of integration of the unselected parent among the integrated viral sequences in rat transformants.

detection level. As summarized in Table 1, the high level of mutant to wild type did not alter the results: only one in 14 transformants analyzed from this infection contained the unselected parent.

Similar results were obtained in further experiments: for example B2 was recovered in no transformant of three analyzed in experiment 2b performed at a 5:1 ratio of mutant to wild type, and only in three of 12 cases from experiment 4, carried out at a ratio of 20 to 1. As can be seen in Table 1 the recovery of the unselected parent seems independent of the total multiplicity of infection which ranged from 1:1 (experiment 2a) to 10:10 (experiment 5a,b) to 20:1 (experiment 3, 4). Fig. 3 illustrates that the ratio of mutant to wild type genome in the infected cells in experiment 4 is indeed as high as expected from the ratio of the two parents in the infection mix, as established by using plaque-forming units. The DNA shown in the figure was extracted 3 days postinfection [a time at which there has been little or no replication of the input genomes (19)] and was digested with PvuII and hybridized with the pPyH4 probe which spans the region deleted in mutant B2. The 0.94 kb deleted PvuII fragment is clearly in vast excess over the wild type 1.2 kb fragment.

Low probability of recovery of two parental genomes was also demonstrated in transformants derived from mixed infections with transformation defective HOP1033 and transformation competent ts3. In these, only 9 of 69 transformants analyzed contained both parental genomes (17).

Underrepresentation of the mutant sequences in mixed transformants. In those few instances in which the unselected parent was recovered in the transformed cells, the level of sequence from the deletion parent was not

Fig. 3. Ratio of wild type and mutant genomes in the infection mix.

Rat F-111 cells (5 x 10^5 cells per 60 mm dish) were infected with a mixture of wild type RA and hr-t mutant B2 at (multiplicities of 1 and 20 respectively) (experiment 4, Table 1). Total DNA was extracted 3 days postinfection, DNA was digested with PvuII and hybridized with pPyH4 as in Fig. 2. The 1.2 kb wild type fragment and 0.94 kb mutant fragment are shown. A: confluent cells (experiment 4a). B: one-tenth of cells shown in A were passed 10 hours postinfection and allowed to grow to confluency (experiment 4b, Table 1).

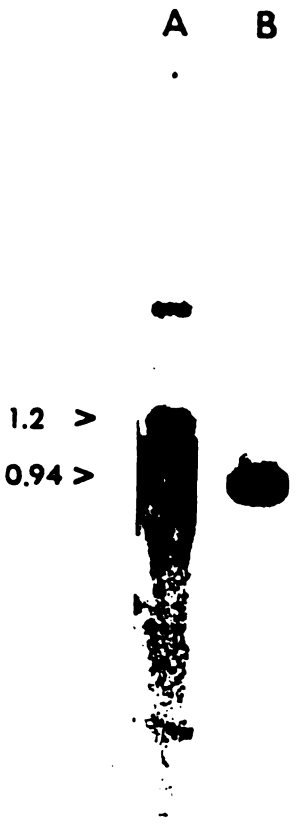


Figure 3. Ratio of wild type and mutant genomes in the infection mix.

represented proportionally to its level in the input. This is illustrated in Figs. 3 and 4 for the three mixed transformants derived from experiment 4, Table 1. Fig. 3 described above shows that the ratio of non-transforming to transforming parent in the infected cells at early times postinfection is very high: i.e. in the 20:1 ratio range established for the infection mix using stock titers. In contrast, those transformants which do carry the nontransforming mutant (Fig. 4) show ratios of wild type to deletion sequence which varies from, at most, equal (lane 1), to much less than equal (lane 3). Similar results were obtained in the other experiments involving high ratios of mutant to wild type (experiment 3, 7, Table 1). The presence of free viral DNA in those transformants does not alter these conclusions since the unintegrated DNA is produced by in situ replication from the integrated sequence (15) and the composition of unintegrated copies is a faithful copy of the integrated sequences (15,17).

The loss of the deletion mutant is not due to underreplication. Persistence of the mutant and wild type sequences in the infected cells was compared from early times after infection until the appearance of transformed foci over the monolayer. For this purpose, F-111 cells were infected at equal multiplicities of the two parents RA and B2 to allow the detection of both parents throughout the course of infection. Furthermore, this experiment was also designed to test that the lack of recovery of the hr-t mutant was not due to segregation to different cells by cell division. Thus, one-half of the plates were maintained at confluency postinfection while the other half were diluted 1 to 30 to allow cells to divide. Total DNA was isolated from infected cells at various times post infection and analyzed by Southern blotting. Results from experiment 5 are shown in

Fig. 4. Underrepresentation of the unselected parent among mixed transformants.

Rat transformants were obtained as described in Fig. 1 except that the ratio of mutant to wild type was 20:1. The three mixed transformants among 12 analyzed from experiment 4 in Table 1 which contained integrated wild type and mutant genomes are shown. High molecular weight DNA was extracted, digested with PvuII and hybridized with pPyH4 as described in Fig. 2. Markers shown are as in Fig. 3.

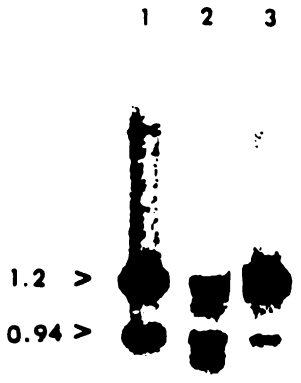


Figure 4. Underrepresentation of the unselected parent among mixed transformants.

Fig. 5. It is clear that the signal from the deletion mutant is retained in the population of infected cells. As is most evident in the infection of confluent cells, the ratio of wild type and mutant is not altered during the course of infection and the mutant persists up to the time of appearance of transformed foci. In the population of dividing cells, the signals of both wild type and mutant DNA decrease, relative to cellular DNA. We have recently demonstrated that a low level of viral DNA synthesis occurs in F-111 cells maintained at 37°C (19). Clearly, the amount of synthesis does not ensure efficient synthesis and segregation of the viral genomes in daughter cells, so that under conditions of low cell density, the signal is lost after a few generations. However, there is no preferential loss of the deletion parent in these conditions. If anything the B2 genome is preferentially retained. This may be due to differences in the enhancer region between the two parents (M. C. Chen and M. M. Fluck, in preparation).

The loss of the nontransforming deletion mutant is not due to a segregation problem. Most of the experiments described above were carried out in conditions designed to optimize transformation. These usually allow for a few cell divisions post infection before the monolayer becomes confluent. Since the time at which the integration events are fixed is not known, and may not be during the first generation, we considered the possibility that the transformation-defective hr-t mutant and wild type might segregate to different cells during cell division, resulting in loss of the hr-t mutant from cells which contain wild type and can potentially become transformed. Thus, infections were carried out with monolayers derived from exponentially growing cells reseeded at confluency prior to infection

Fig. 5. Persistence of the viral genome in the infected cell population. Confluent F-111 rat cells were infected as described in Materials and Methods (experiment 5, Table 1), with transforming parent RA and non-transforming parent B2 at multiplicities of ten for each parent. One half of the plates were maintained at confluency (lanes 1 to 3) while the other half (lanes 4 to 7) were diluted 1 to 30 to allow cells to divide. Total cellular DNA was isolated at the times indicated (dpi = days postinfection), digested with PvuII, electrophoresed and analyzed by Southern blotting and hybridization as described in Materials and Methods and in Figs. 1 to 3. Hybridization was carried out with pPyH4 as described in legend to Fig. 2.

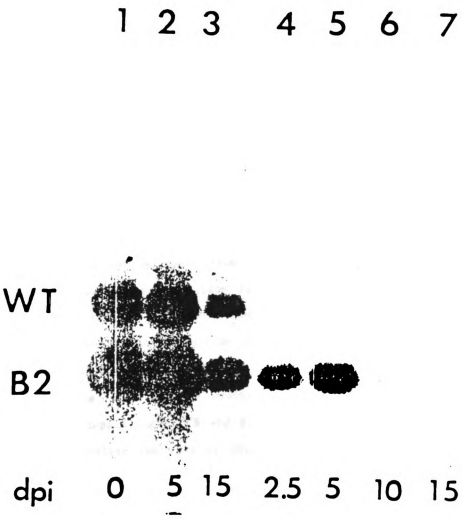


Figure 5. Persistence of the viral genome in the infected cell population.

(experiment 4a and 5a, Table 1). As summarized in Table 1 and illustrated in Fig. 6, for nine transformants from experiment 5, this procedure did not increase the recovery of the hr-t mutant. Of 42 transformants analyzed in experiments 4 and 5, four in 18 contained the hr-t mutant in the infections with nondividing cells, versus six in 24 from the dividing cells. As already noted above, in mixed transformants the ratios of the intensity of mutant to wild-type fragment were considerably in favor of the wild-type fragment even for those transformants which arose in the nondividing condition. These experiments suggest that integration (or the events which fix it) occur early after infection.

The underrepresentation of unselected genomes is a general phenomenon. We considered the possibility that the underrepresentation of the hr-t mutant B2 in transformants derived from mixed infection with strain RA is specific to this particular pair of genomes. For example, differences in adsorption, decapsidation, histone acetylation (25), might either retard one of the parents or segregate them to separate cell compartments. Different affinities for transcription factors or large T-antigen could also differentiate their integration potential. This possibility needs to be entertained since RA and B2 differ in multiple sites: the origin-enhancer (K. Higgins, K. Friderici, M. C. Chen, and M. M. Fluck, unpublished), in the large T-antigen coding region (between EcoRI and NsiI) (18), and in the capsid region (6 and K. Friderici and M. M. Fluck, unpublished). To analyze the role of differences in the origin-enhancer region (encompassing binding sites for the large T-antigen and transcriptional factors), a strain was reconstructed in which the small BglI-DclI fragment comprising the origin-enhancer region in strain RA was replaced by

the equivalent region from mutant B2. As can be seen (experiment 6, Table 1), this exchange did not relieve the underrepresentation of B2, as B2 was not recovered in any of 12 transformants analyzed. To further test the generality of the underrepresentation, other pairs of parental strains were used including wild type A2 and other hr-t mutants such as 30'b and 3A3 (experiments 7 and 8, Table 1) or wild type A3 and mutant d145 (see below). As can be seen, the unselected parent was also underrepresented in these infections.

Evidence that the selected and the unselected parent are in the same cells. One can imagine that the underrepresentation of the unselected parent in the transformed cells reflects a low frequency of co-infection which may be linked to an exclusion phenomenon. This possibility is not ruled out by the persistence of viral genomes of both types in the infected cells, since these might reside in different cells. However, a physiological aspect of the co-infection suggests that this is not the case. When Fischer rat F-111 cells are co-infected with RA and hr-t mutants such as B2, the yield of transformants in the mixed infection is higher than those observed in infection with wild type alone used at the same multiplicity. This effect is dependent on the dose of B2 mutant present in the infection. As is illustrated in experiment 2, Table 1, the yield of transformants was increased 4-fold at a 1 to 1 ratio or 17-fold at a 5:1 ratio. We are assuming that this effect is due to complementation by the B2 parent of a large T-antigen mutation which was very recently discovered in strain RA (18). Similar examples of complementation have been documented previously between large T-antigen and middle T-antigen mutants (9). It is worth emphasizing that even when a high level of complementation is

Fig. 6. Recovery of an unselected viral genome from transformants obtained from nondividing or dividing infected F-111 rat cells. Infections of F-111 rat cells were carried out as described in the legend to Fig. 5 and Materials and Methods. Transformants were isolated and high molecular weight DNA extracted and analyzed as described in legends Fig. 1 to 3. DNA digestion with PvuII and hybridization were as described in legend Fig. 2. Lanes 1 to 4 were from cells maintained at confluency postinfection. Lanes 5 to 9 were diluted 1 to 30 postinfection.

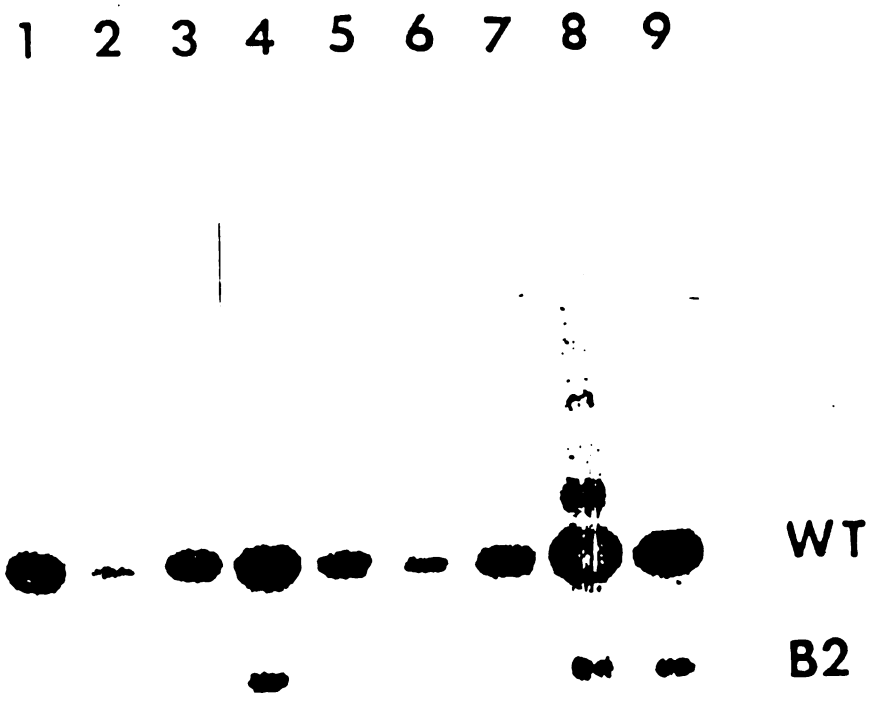


Figure 6. Recovery of an unselected viral genome from transformants obtained from nondividing or dividing infected F-111 cells.

observed, the complementing parent is underrepresented in the transformant. In experiment 2b of Table 1, a 17-fold complementation factor was observed in co-infections with B2, at a 5:1 ratio; yet the mutant was not recovered in three transformants analyzed.

In contrast, when co-infections are carried out with wild type A2, the yield of transformants is depressed proportionally to the dose of hr-t mutant. Depression of transformation was recorded in experiments 7 and 8, Table 1. For example, in experiment 6, the yield of transformants obtained in co-infection of wild type A2 with hr-t mutant 3A3 was reduced five times compared to the yield obtained with the same dose of wild type in single infection. This effect has also been noted previously and referred to as a dominant lethal effect (9). We believe that its potential origin lies in competition for a limited factor (M. C. Chen and M. M. Fluck, in preparation). At very high levels of hr-t mutant B2 (beyond the complementing range), a depression of transformation is also observed in mixed infections between RA and B2 (M. C. Chen and M. M. Fluck, in preparation).

The depressive effect (competition) of hr-t mutants on wild type transformation and the positive effect (complementation) of these same hr-t mutants on strain RA transforming potential reflect a complex biological system with multiple levels of interaction. It is worthwhile pointing out that, in mixed infections between hr-t middle T-antigen- and ts-a large T-antigen-deficient mutants, similar complex interactions are observed (9). When the mixed infections are carried out at 33°C, a situation in which the ts-a mutant can transform, a depression of transformation is observed in the mixed (ts-a + hr-t) infection, compared to infection with ts-a alone, indicative of what we believe to be competition for a limited factor. In

contrast, when the mixed infections are carried out at 39°C, a situation in which the ts-a mutant cannot transform, an enhancement (complementation) is observed in the mixed infection (ts-a + hr-t) compared to infection with ts-a alone (9). In this case, complementation of the large T-antigen defect of the ts-a mutant by the functional large T of the hr-t mutant, is stronger than the competition between the two strains for limiting factor(s) and net complementation is observed. Experiments in progress suggest that the differences in competition between A2 + B2 and RA + B2 co-infections may be due to differences in the enhancer region between these two strains (M. C. Chen, and M. M. Fluck, unpublished), in addition to the complementable RA defect in large T-antigen noted above.

In summary, we believe that both the cooperation and the depressive effect in the co-infection reflect the fact both parental genomes are co-infecting the same cells.

The reductive effect of mutant B2 on wild type transformation opens the possibility that in the population of infected cells only those with relatively high ratios of wild type to mutant can become transformed, thus providing an explanation for the rare recovery of the transformation-defective mutant genome in transformed cells. However, this explanation is not applicable to the infections performed with strain RA.

Underrepresentation of an unselectable genome is also observed in hamster transformants. To analyze whether the low frequency of co-integration of two parental genomes in transformed cell can be generalized to different hosts, we repeated the experiment using the other nonpermissive host for polyoma transformation, i.e., the hamster. Baby hamster kidney (BHK) cells were infected with mixtures of strains RA and B2

(experiment 10, Table 1). Thirteen transformants were analyzed in detail. Only one of these contained the B2 parent in addition to the wild type (Fig. 7).

In contrast to the results described above for F-111 transformants, the integration patterns of the BHK transformants were unusual. Ten out of 13 transformants analyzed contained a single viral insertion site with an incomplete copy of the viral genome including a deletion of the carboxy terminus of large T-antigen. Only two transformants in 13 contained multiple sites of integration with tandem repeats of the viral genome. The BHK transformant in which the unselected parent was recovered is one of the two transformants for which "typical" integration patterns were observed. The basis for the differences between the BHK and Fischer rat integration pattern in mixed infections with strain RA is not fully understood. Further experiments demonstrated usual integration patterns for strain RA and A2 in single infections of BHK cells (K. McWilliams and M. M. Fluck, unpublished). The tendency to inactivate large T-antigen in transformants has been noted before (7). We believe that an unusual selection pressure was applied during the selection of the BHK transformants (selection of very well-developed foci, followed by recloning in agar), which may have selected for a very early inactivation of large T-antigen.

Low probability of double integration events in transformants derived from mixed infections with two selectable genomes. The experiments described above all make use of hr-t deletion mutants whose integration is not selected in the transformation process. We considered the possibility that the underrepresentation of hr-t genomes represents a problem specific to all hr-t mutants. This hypothesis is not highly likely since mixed

Fig. 7. Underrepresentation of an unselected viral genome in hamster transformants.

Baby hamster kidney (BHK) cells were infected as described in legend to Fig. 1 and Materials and Methods. Transformants were isolated and analysis is identical to legend to Fig. 2. Digestion is with MspI and hybridization with pPyH4.

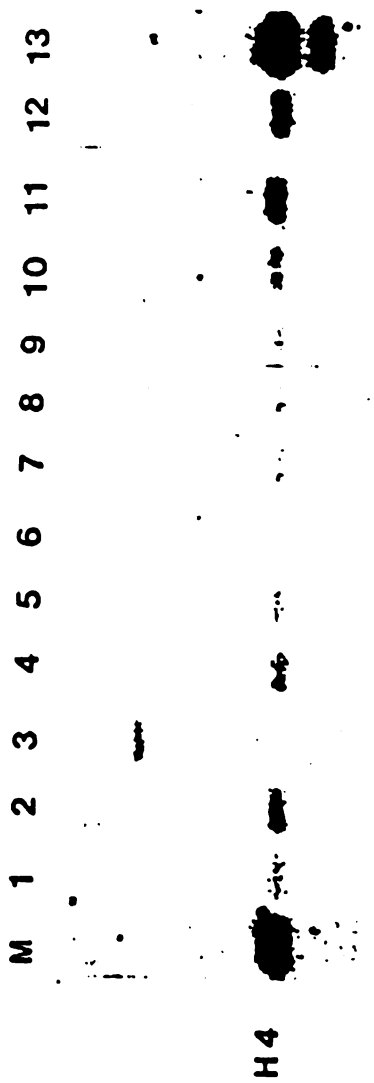


Figure 7. Underrepresentation of and unselected viral genome in hamster transformants.

infections with two non-overlapping hr-t mutants generate transformants by recombination at relatively high frequency (compared to wild type infection) (S. Kalvonjian, C. Priehs, H. H. Chen, and M. M. Fluck, manuscript in preparation). However, differences in capsid phosphorylation (14) and in chromatin acetylation (25) have been documented between the hr-t mutant and wild type, and it is conceivable that these might lead to differences in processing by the host. To determine whether the low recovery of a second parental genome is specific for transformation-deficient hr-t mutants, we set up a mixed infection with an isogenic pair of strains in which both parents can transform and which can also be distinguished from each other by restriction endonuclease analysis. We chose wild type A3 and a deletion mutant, d145, which was isolated from A3. Thus, these two strains have identical sequences, except for the d145 deletion of 66 bp in the overlapping middle T-antigen-large T-antigen reading frames (2). This deletion can be detected by digestion with SstI and observation of fragment 3. The d145 mutation does not result in a noticeable transformation nor growth deficit (2). Thus, this pair of strains offers a further advantage in our analysis since both genomes are selectable. The result of two experiments (#9) are presented in Table 1 and one is presented in Fig. 8. In one of these experiments (9b shown in Fig. 8), infected cells were passed to agar after infection, to prevent cell division and segregation of the two parental genomes to different cells. In the latter experiment, cells were infected at a multiplicity of 10 each of the two parental viruses. Analysis of the viral genomes in the imput is in agreement (not shown). Transformation frequencies in the mixed infection and in the controls were very close (1.3% for the infection with A3, 0.6% for d145 and

Fig. 8. Low probability of double integration events in transformants derived from mixed infections with two transforming strains.

F-111 rat cells were infected with wild type A3 and transformation competent deletion mutant d145 at a multiplicity of infection of 10 p.f.u. of each parent (experiment 9, Table 1). Transformants were isolated and high molecular weight cellular DNA was analyzed as described in legend of Fig. 1 and 2. Digestion is with SstI and hybridization to a probe for the whole viral genome (pPy-1).

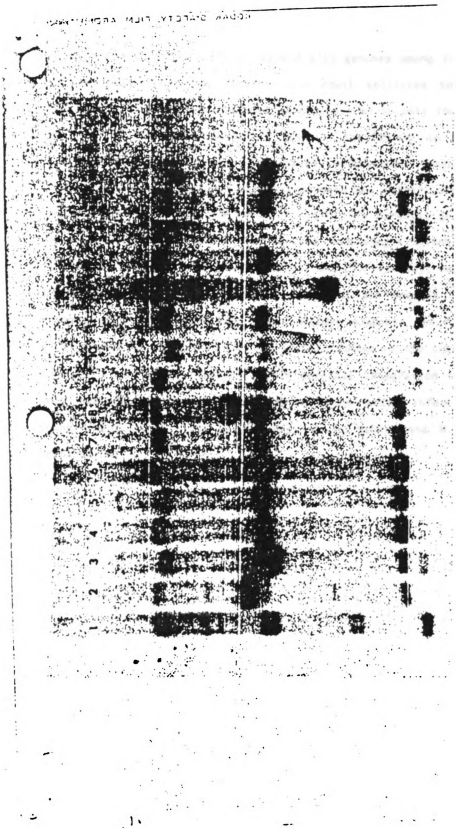


Figure 8. Low probability of double integration events in transformants derived from mixed infections with two transforming strains.

1.8% for the mixed infection), supporting the notion that A3 and d145 transform equally efficiently. Twenty-two transformants were analyzed in detail; all showed normal patterns of integration. In eight transformants, the A3 wild type genome was present alone, in 10, the d145 genome alone was found. The equal recovery of A3 and d145 genomes among the transformants suggest that these two strains have equal abilities to integrate and transform. Both parental genomes were recovered in only four transformants of the 22 twelve analyzed. Results are shown in Fig. 8 for 14 transformants derived from the mixed infections, including the four transformants in which both parental genomes are integrated. Note that in all four cases, one of the level of one of the parental genomes is higher than the other. One clone each derived from single infection with either A3 or d145 alone is also shown (lanes 1 and 2). Finally, note that all the fragments expected after digestion with SstI are present (except for one fragment missing in lane 12). This pattern supports the finding that the viral genome is integrated in head to tail tandem in the transformants. In agreement with these results, double parental integration was detected in only one of 23 transformants derived from mixed infections with two transforming strains Py3-33 and ts3 (D. L. Hacker and M. M. Fluck, in preparation).

Discussion

The experiments presented above were designed to analyze how two independent viral genomes integrate into the host chromosome during the normal process of neoplastic transformation of rat and hamster cells by polyoma virus, i.e., a context in which integration of a at least one viral genome is known to occur. For this purpose, rat transformants were derived from mixed infections with wild type virus (parental genome whose integration is selected) and a nontransforming deletion mutant (hr-t mutant) (whose integration is not selected). The recovery of the non-selected parent in the transformants was analyzed by restriction endonuclease digestion of cellular DNA followed by electrophoresis and hybridization, using appropriate endonucleases, which resolve the two parental genomes. Our results are as follows:

The overall integration pattern of transformants derived from such infections are typical: i.e. each transformant displays head-to-tail tandem structures of the viral genome, apparently integrated at multiple sites in the host genome. This integration pattern is compatible with the occurrence of normal integration events in these mixed infections. As expected, all transformants contain the wild type transforming parent, since selection for the retention of that parent was applied by selecting for transformation. Surprisingly, the unselected parent is vastly under-represented among the transformants, at least that fraction of the viral genome encompassing the deletion marker mutation. Among 108 transformants analyzed from mixed infections using an unselectable second parental genome, the unselected genome was recovered in only 12 cases. Forty percent of the transformants were derived from infections using equal

amounts of the two parents while the remainder 60 percent of the transformants analyzed were obtained from infections in which the unselectable parent was used in considerable to high excess over the transforming selectable parent (a condition used in 40 percent of the infections). This increase of the ratio of unselectable to selectable parent in the infection did not increase the probability of recovering the unselected parent in the transformants. Furthermore, in those transformants in which the unselected parent was recovered, the ratio of mutant to wild type sequences was at most equal, even though some were derived from infections performed at an extreme ratio of mutant to wild type (200:1 or 20:1).

Control experiments demonstrated that the hr-t mutant genomes persist as well as the wild type in the infected cells, suggesting that the mutants have no major adsorption--decapsidation--replication problem. Previous experiments have demonstrated that an hr-t like mutant integrates normally (20), as do the recombinants generated in crosses between 2 nonoverlapping hr-t mutants (S. Kalvonjian, C. Priehs, and M. M. Fluck, manuscript in preparation). The presence of both mutant and wild type genomes in the same cells early in infection is a prerequisite to any conclusion regarding the integration of the two genomes in the same cell. This was insured by infecting confluent cells which were not allowed to divide between the time of infection and transformation or by passing the infected cells into a semi-solid agar suspension. Furthermore, biological features of the mixed infection indicated that co-infection was taking place since mixed infections with transforming and hr-t mutants lead to an increase in transformation frequency when the transforming parent was RA or, conversely, a decrease in transformation when the transforming parent was wild type A2.

This strongly suggests that both parental genomes were in the same cell and in one case with strain RA, complementing each other. The apparently opposite effect of the hr-t parent in mixed infection with wild type A2 compared with pseudo wild type RA has been addressed in details in the results section. The possibility that cells cannot become transformed when both the wild type and the transformation defective parents have integrated appears highly unlikely. Multiple examples of complementation in the process of transformation have been demonstrated previously in mixed infections with large T-antigen mutants and hr-t mutants (9). Furthermore, we have previously described a highly transformed cell line which contains the hr-t mutant in addition to a single copy of the wild type middle T-antigen gene (23), as well as cases of transformants from mixed infections which express a wild type and a deleted middle T-antigen protein (9). Thus, the presence of both wild type and hr-t mutant alleles in the same cell does not prevent transformation. In summary, it appears that both the transforming and the non-transforming parent infect the same cells, and persist equally in those cells during the course of infection. Yet, the frequency of recovery of the two parents in cells selected for the presence of the transforming parent (i.e. transformed cells) is low. The nontransforming parent is highly underrepresented in those cells. This underrepresentation is not specific to any pair of strains used. In fact, it is not peculiar to the recovery of an unselectable genome, since a second selectable genome was only recovered in six cases of 36 transformants selected from a mixed infection carried out with two transforming parent strains. The underrepresentation of a second parental genome in transformants derived

from mixed infection was observed in the two cell lines tested i.e., in the F-111 Fischer rat and in the BHK hamster cell lines.

Interestingly, the frequency at which a second parental genome is recovered is not affected by the number of times the infected cells are allowed to divide after infection before the selection for transformation is applied. This suggests [as do other unpublished results from our lab and previous results (13)] that the integration events are fixed early postinfection.

The present results do not fit the expectations from the simplest model of integration reviewed in the Introduction. The analysis of integration pattern of viral genomes in a large collection of transformants, derived both from polyoma virus and SV-40 infections of nonpermissive cells, has suggested that the viral genome can integrate at a large number of sites in the host genome (4,5). Judged by restriction endonuclease analysis, multiple integration events have usually occurred within a single transformant (8-10 as reviewed in the Introduction). The simplest model would be that a large number of sites are available for the integrating viral genomes and that each integration event within a single cell in the transformation pathway occurs independently from the other integration events within the same cell. Thus, in transformants derived from mixed infections, we expected to find both parental genomes integrated at independent sites and we expected them to be represented within a single transformant proportionally to their ratio in the infection mix. As judged by the usual criteria of multiple bands in digests with enzyme which do not cut the viral genome, multiple integration sites are apparently present-an example with seven apparent sites is shown-; however, the nonselected non-

transforming parent is very strongly underrepresented in the population of integrated viral genomes in the transformants. These results can be interpreted to mean that viral integration is infrequent as suggested in the past (28) based on the low frequency of stable transformants. Such an interpretation contradicts the picture based on Southern blot analysis. Low frequency of integration could be due to limited amounts of host factor(s) involved in integration or to limited numbers of sites in the host chromosome available for integration. Some indirect support for the former hypothesis is available. The progressive depression of transformation in response to increasing doses of transformation deficient mutant in co-infection with wild type is compatible with competition for a limited factor, or a limited site, or both. The factor could be an enhancer binding factor required for the transcription-replication of the viral genome and linked to its integration (22). We are presenting data elsewhere which support a role for the enhancer in integration (M. C. Chen and M. M. Fluck, manuscript in preparation). However, in co-infections with pseudo-wild type RA, the sufficient levels of factors are present to allow replication of the nonselected (B2) parent, present in excess and yet integration of B2 is rare, points towards a limit for integration, separate from a limit on replication. Possibly integration sites (or a structure linked to them) are limited. Or the probability of integration is low. The latter is compatible with the low frequency of stable transformation by polyoma virus, and suggests by comparison with the frequency of abortive transformation, that integration may be a rate limiting step in transformation (28).

If the above explanation is correct, we must reinterpret the apparently elevated number of integration sites revealed in previous analyses of integration patterns and reviewed above. Preliminary results from our lab suggest that at least part of the multiple bands obtained in digests with enzymes which do not cleave the viral genome may consist of multiple forms of the same integration site (K. Friderici, L. J. Syu, and M. M. Fluck, unpublished) and we are testing the hypothesis that these are produced by in situ replication of the integrated viral genomes.

Recent experiments from our lab (6) have demonstrated a high level of interviral recombination in the viral genomes which become integrated in the transformed cells. The present experiments are not in contradiction with these results since in most cases analyzed, the integrated recombinant viral genomes have undergone recombination in two adjacent to intervals, generate a genome such that for any given interval the sequences of only one of the parent are present. Thus, in the case of the present experiments, the wild type sequence must be selected over those of the hr-t mutant. Interestingly in a few cases analyzed so far in which both wild type and hr-t mutant sequences were recovered in the same transformants, these were present at the same site demonstrating double parental integration by an interviral recombination event which involves a single integration into the host chromosome (8,12).

In conclusion, the experiments presented above suggest that in the neoplastic transformation of rat cells by polyoma virus a highly limited number of viral genomes become integrated. This number appears more restricted than either the number of viral genomes which can be transcribed since complementation or competition can be observed, or that which can be

replicated (since both parents persist). It is possible that the viral genomes which serve as templates for transcription and replication are not in the same compartment as those which become integrated. Thus, it may be that there are integration centers, at which multiple genomes interact since high recombination has been observed among those integrated viral sequences, and the number of integration centers is highly limited.

LITERATURE CITED

1. **Basilico, C., S. Gattoni, D. Zouzias, and G. Della Valle.** 1979. Loss of integrated viral DNA sequences in polyoma-transformed cells is associated with an active viral A function. Cell 17:645-659.
2. **Bendig, M. M., T. Thomas, and W. R. Folk.** 1980. Viable deletion mutant in the medium and large T-antigen coding sequences of the polyoma virus genome. J. Virol. 33:1215-1220.
3. **Benjamin, T. L.** 1970. Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. USA. 67:394-399.
4. **Birg, F., R. Dulbecco, M. Fried, and R. Kamen.** 1979. State and organization of polyoma virus DNA sequences in transformed rat cell lines. J. Virol. 29:633-648.
5. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangement of simian virus 40 sequences in DNA of transformed cells. Cell 9:269-287.
6. **Carmichael, G. G., and T. L. Benjamin.** 1980. Identification of DNA sequence changes leading to loss of transforming ability in polyoma virus. J. Biol. Chem. 255:230-235.
7. **Fluck, M. M., and T. L. Benjamin.** 1979. Comparisons of two early gene functions essential for transformation in polyoma virus and SV-40. Virology 96:205-228.
8. **Fluck, M. M., R. Shaikh, and T. L. Benjamin.** 1983. An analysis of transformed clones obtained by coinfections with Hr-t and Ts-a mutants of polyoma virus. Virology 130:29-43.
9. **Fluck, M. M., R. J. Staneloni, and T. Benjamin.** 1977. Hr-t and ts-a: two early gene functions of polyoma virus. Virology 77:610-624.

10. Freeman, A. E., R. V. Gilden, M. L. Vernon, R. G. Wolford, P. E. Hugunin, and R. J. Huebner. 1973. 5-Bromo-2-deoxyuridine potentiation of transformation of rat embryo cells induced in vitro by 3-methylcholanthrene: induction of rat leukemia virus gs antigen in transformed cells. Proc. Natl. Acad. Sci. USA. 70:2415-2419.

11. Feunteun, J., L. Sompayrac, M. M. Fluck, and T. L. Benjamin. 1976. Localization of gene function in polyoma virus DNA. Proc. Natl. Acad. Sci. USA. 73:4169-4173.

12. Friderici, K., S. Y. Oh, R. Ellis, V. Guacci, and M. M. Fluck. 1984. Recombination induces tandem repeats of integrated viral sequences in polyoma-transformed cells. Virology. 137:67-73.

13. Fried, M. 1965. Cell transforming ability of a temperature-sensitive mutant of polyoma virus. Proc. Nat. Acad. Sci. USA 53:486-491.

14. Garcea, R. L., K. Ballmer-Hofer, and T. L. Benjamin. 1985. Virion assembly defect of polyomavirus hr-t mutants: Underphosphorylation of major capsid protein VP1 before viral DNA encapsidation J. Virol. 54:311-316.

15. Gattoni, S., V. Colantuoni, and C. Basilico. 1980. Relationship between integrated and nonintegrated viral DNA in rat cells transformed by polyoma virus. J. Virol. 34:615-626.

16. Griffin, B. E., M. Fried, and A. Cowie. 1974. Polyoma DNA: a physical map. Proc. Natl. Acad. USA. 71:2077-2081.

17. Hacker, D. L., and M. M. Fluck. 1989. Hyperelevated and segregated recombination in the integration pathway of polyomavirus. Mol. Cell. Biol. 9:995-1004.

18. **Hacker, D. L., and M. M. Fluck.** 1989. A non-lethal mutation in large T-antigen of polyomavirus which affects viral DNA synthesis. J. Virol. 63:776-781.

19. **Hacker, D. L., and M. M. Fluck.** 1989. Studies of viral DNA synthesis in nonpermissive rat F-111 cells and its role in neoplastic transformation by polyomavirus. Mol. Cell. Biol. 9:648-658.

20. **Lania, L., M. Griffiths, B. Cooke, Y. Ito, and M. Fried.** 1979. Untransformed rat cells containing free and integrated DNA of a polyoma nontransforming (Hr-t) mutant. Cell 18:793-802.

21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

22. **Muller, W. J., D. Dufort, and J. A. Hassell.** 1988. Multiple subelements within the polyomavirus enhancer function synergistically to activate DNA replication. Mol. Cell. Biol. 8:5000-5015.

23. **Priehs, C., K. Friderici, L. Winberry, and M. M. Fluck.** 1986. Properties of cells transformed by the middle T-antigen-coding region of polyomavirus. J. Virol. 57:211-218.

24. **Ruley, H. E., and M. Fried.** 1983. Sequence repeats in a polyoma virus DNA region important for gene expression. J. Virol. 47:233-237.

25. **Schaffhausen, B. A., and T. L. Benjamin.** 1976. Deficiency in histone acetylation in nontransforming host range mutants of polyoma virus. Proc. Nat. Acad. Sci. USA 73:1092-1096.

26. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

27. **Staneloni, R. J., M. M. Fluck, and T. L. Benjamin.** 1977. Host range selection of transformation defective hr-t mutants of polyoma virus. Virology 77:598-609.

28. **Stoker, M., and R. Dulbecco.** 1969. Abortive transformation of the TsA mutant of polyoma virus. Nature 223:397-398.

29. **Winberry, L. K., C. J. Stewart, B. S. Schaffhausen, and M. M. Fluck.** 1985. Transformation by polyoma ts-a mutants. I. Characterization of the transformed phenotype. Virology 144:433-447.

MICHIGAN STATE UNIV. LIBRARIES
31293006292571