TRANSCRIPTIONAL REGULATION OF HUMAN NEUROTROPIC PAPOVAVIRUS, JCV

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TRANSCRIPTIONAL REGULATION OF HUMAN NEUROTROPIC PAPOVAVIRUS, JCV

By

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A thesis submitted to the school of graduate studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Division of Basic Medical Sciences
Faculty of Medicine
Memorial University of Newfoundland

April, 1994

St. John's Newfoundland

ISBN 0-315-96051-5

This thesis is dedicated to
my parents

Smt. K. Rukmini
and
Sri. K.V. Ranganatham

ABSTRACT

The human papovavirus JC virus (JCV) replicates only in glial cells and exhibits strict cell-specificity for expression. JC virus (JCV) has been frequently found in the plaques of brains of victims of the fatal demyelinating brain disease, progressive multifocal leukoencephalopathy (PML). PML is associated with immunodeficiency and has been identified recently as a frequent complication of acquired immunodeficiency syndrome (AIDS). The regulatory region of JCV is contained mainly within the two 98 base pairs (bp) tandem repeats and is functional in both directions, with the early and late promoter-enhancer (JCV_E and JCV_L) controlling expression of large and small tumour antigens and viral structural proteins, respectively.

Previously, it was shown in our laboratory that the regulatory region of JCV confers glial cell-specificity to JCV and contains three potential nuclear factor 1 (NF1) motifs (Nakshatri et al, 1990a). One motif towards the late side of the genome has a 3 bp palindromic repeat and the other two located within the 98 bp repeats have 6 bp inverted palindromic sequences.

My initial objective was to evaluate the functional role of NF1 motifs in the restricted cell-specificity of JCV. This was approached by site-directed mutagenesis of the nuclear factor 1 (NF1) motifs within the viral regulatory region. The

NF1 motifs, located within the 98 bp tandem repeats which contain 6 bp perfect inverted palindromic sequences were important for glial cell-specific expression of JC virus in differentiated embryonal carcinoma cells in vivo. The NF1 site on the late side of the repeats was not important, an observation confirmed by in vitro transcription studies. These observations were correlated with in vitro DNase I footprinting and mobility shift assays, which demonstrated specific interactions of factors in glial cell nuclear extracts with NF1 sites.

To characterize the brain-specific factor(s) inducing the expression of JCV, a cDNA encoding the factor binding to NF1 II/III located in the repeats was isolated by screening the cDNA library prepared from retinoic acid-differentiated P19 embryonal carcinoma cells by Southwestern blotting. Co-transfection of cDNA with JCV_E and JCV_L reporter plasmids resulted in the expression of JCV in nonglial cells such as HeLa cells. These results suggested that NF1 II/III plays a key role in the glial cell type-specificity of JCV.

Mutations in all the three potential NF1 binding sites greatly reduced the transcriptional activity of JCV early promoter-enhancer in glial cells. However, a residual greater than basal level of activity was still observed. Examination of the JCV promoter-enhancer sequences revealed a cyclic AMP response element (CRE) motif, TGAGCTCA, 4 bp from the NF1

II/III binding site in the repeats. This increased the transcriptional activity of JCV_E after treatment with the cAMP analogue dibutyryl cAMP and cyclic AMP inducer forskolin. Mutations in CRE motifs abolished the induction of JCV expression by cAMP. In vitro binding studies showed the interaction of an approximately 43 kDa protein with CRE oligonucleotide. The results indicated an additive effect of NF1 II/III and CRE in the glial cell-specific expression of JCV.

JCV large T-antigen functionally transactivates the viral late promoter only in glial cells and the molecular mechanism for transactivation has remained elusive. The NF1 sequences of JCV and the amino acids of JCV T-antigen required for the transactivation have remained unclear. I performed experiments to address these issues. Using site-directed mutagenesis of NF1 sites of JCV_L, the integrity of nuclear factor 1 (NF1) binding motifs in the 98 bp repeats (NF1 II/III), but not the motif external to the repeats, was found to be essential for the induction of JCV_L activity by JCV T-antigen, a result which was confirmed by in vitro transcription assays. Mobility shift assays detected the increased binding of NF1 to the NF1 II/III sequences in the presence of T-antigen. This suggests that JCV T-antigen facilitates the increased binding of NF1 to NF1 II/III site, thereby activating the late promoter of JCV. A model

describing the mechanism has been proposed.

The region(s) of T-antigen necessary for the transactivation of JCV_L was assessed with a series of constructs containing mutated coding sequences which I generated. The amino acids 196-437 region of JCV T-antigen was required for most of the transactivation of JCV_L and transformation of primary baby rat kidney cells.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisors Drs Mary M. Pater and Alan Pater for their valuable and immense guidance. I wish to thank Drs. Gary Paterno and Peter Howard for their guidance as supervisory committee members for various durations. I am grateful to Dr. Chet Michalaski, former assistant dean and Dr. Verna Skanes, present assistant dean for their moral support and encouragement. I would like to thank Drs. Jon Church, Laura Gillespie and Martin Mulligan for serving as members of my comprehensive examination committee.

I am grateful to Sri. Venkateswarlu, elementary school teacher and late Sri. G. Venkata Reddy, Head master of high school studies for their initial guidance that enabled me to work efficiently and in a planned manner. I am bereft of words to express my gratitude to Dr.S. Krishna Swamy, former professor, department of Microbiology, College of Veterinary Sciences, Tirupati for his moral support which inspired me to pursue higher studies. I am also grateful to Dr. P.C. Choudhury and Dr. P. Rama Rao, professors, college of Veterinary Sciences, Tirupati for their help.

I am thankful to Dr. Harikrishna Nakshatri for his help during the beginning of my Ph.D programme. I am grateful to Dr. Belaguli N. Swamy and Dr. Rakesh Mittal for their stimulating discussions and encouragement and Dr. Kasinadhuni

Satish for his encouragement and nice company. I thank my colleagues, Miss Christine Kisaka, Dr. Khare, Dr. Kouichiro Tsutsumi, Dr. Masatoshi, Mr. Mingfeng Liu, Dr. Nakao, Dr. Sun, Dr. Raghuram, Dr. Shindoh and Mr. Yang. I am especially thankful to Dr. Balaji Balasa for his immense help in making figures and Dr. Devireddy for his help in Western blotting.

The technical assistance provided by Michelle Power, Anne Roche, Clarann Floyd, Masuma Rahimtulla and Ge Jin is highly acknowledged. The support and help extended by Dr. Kota Prasad during my preparation for comprehensive examination is unforgettable. I also thank Drs Nagendra Prasad, Panduranga Rao and Shyam for their help. I thank Mr. Jason Lilly for proof-reading my thesis. I am grateful to Dr. C.V.V Prasad and Smt. C. Sarada and Dr. Markandeya Jois for making me feel at home during the initial part of my programme.

The blessings of my parents, affection of my brothers, best wishes of my sisters-in-law and longstanding waiting and understanding of my fiance Dr. B. Padmalatha has given me the strength, enthusiasm and encouragement in carrying out this study. The financial assistance provided by graduate studies, Memorial University of Newfoundland was of great help to me and is highly acknowledged.

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α-MEM	=	Alpha modification of Eagle's medium
AIDS	=	Acquired Immunodeficiency syndrome
AP1	=	Activator protein 1
AP2	=	Activator protein 2
ATF	=	Activating transcription factor
ATP	=	Adenosine triphosphate
BCIP	=	5-bromo-4-chloro-3-indolyl phosphate
BKV	=	BK virus
bp	=	Base pairs
BRK	=	Baby rat kidney
BRL	=	Bethesda Research Laboratories
cAMP	=	Cyclic Adenosine monophosphate
CAT	=	Chloramphenicol acetyl transferase
CCG1	=	Cell cycle gene 1
cDNA	=	Complementary deoxyribonucleic acid
CIP	=	Calf intestinal phosphatase
CK	=	Casein kinase
CMV	=	Cytomegalovirus
CNS	=	Central nervous system
cpm	=	Counts per minute
CRE	=	Cyclic AMP response element
CREB	=	CyclicAMP response element binding protein
CTD	=	Carboxy terminal domain
CTF	=	CCAAT transcription factor

DBF = TFIID binding factor

DBH = Dopamine β hydroxylase

DMSO = Dimethylsulfoxide

DNA = Deoxyribonucleic acid

DNaseI = Deoxyribonuclease I

DTT = Dithiothreitol

E1A = Early region 1A

EDTA = Ethylenediaminetetraacetic acid

EGTA = Ethylene glycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid

FSK = Forskolin

GFAP = Glial fibrillary acidic protein

GR = Glucocorticoid receptor

GTF = General transcription factor

HEK = Human embryonic kidney cells

HEPES = H-(2-hydroxyethyl)-1-piperzinuthanesulfonic acid

HIV = Human immunodeficiency virus

HNF1 = Hepatocyte nuclear factor 1

HPV = Human papillomavirus

HTLV = Human T cell leukemia virus

IE = Immediate early

Ig = Immunoglobulin

JCV = JC virus

JCV_E = JC virus early promoter-enhancer

JCV_L = JC virus late promoter-enhancer

kDa	=	Kilodalton
LCE	=	Lytic control element
LCP 1	=	Lytic control element-binding protein 1
MBP	=	Myelin basic protein
MCK	=	Muscle creatine kinase
MOI	=	Multiplicity of infection
mRNA	=	Messenger ribonucleic acid
NBT	=	Nitro blue tetrazolium
NC	=	Negative cofactor
NF1	=	Nuclear factor 1
NP40	=	Nonidet P-40
nt	=	Nucleotide
Oct	=	Octamer binding protein
PAGE	=	Poly acrylamide gel electrophoresis
PBS	=	Phosphate buffered saline
PC	=	Positive cofactor
PCR	=	Polymerase chain reaction
PHFG	=	Primary human fetal glial cells
PIC	=	Preinitiation complex
Pit	=	Pituitary specific transcription factor
PKA	=	Protein kinase A
PKC	=	Protein kinase C
PLP	=	Proteolipid protein
PMA	=	Phorbol-12 myristate-13 acetate
PML	=	Progressive multifocal leukoencephalopathy

PMSF	=	Phenylmethylsulfonyl fluoride
pol	=	Polymerase
RA	=	Retinoic acid
Rb	=	Retinoblastoma gene product
RNA	=	Ribonucleic acid
RNase	=	Ribonuclease
rpm	=	Revolutions per minute
RSV	=	Rous sarcoma virus
SDS	=	Sodium dodecyl sulfate
SL1	=	Selectivity factor 1
Sp1	=	Specificity protein 1
SV40	=	Simian vacuolating virus 40
t-antigen	=	Small tumour antigen
T-antigen	=	Large tumour antigen
TAF	=	TBP associated factor
TBP	=	TATA box binding protein
TBS	=	Tris buffered saline
TFII	=	Transcription factor II
TH	=	Tyrosine hydroxylase
TK	=	Thymidine kinase
TLC	=	Thin layer chromatography
TPA	=	12-0-tetradecanoyl phorbol-13-acetate
UD	=	Undifferentiated
UPE	=	Upstream promoter elements
USA	=	Upstream factor stimulatory activity

USF	=	Upstream stimulatory factor
UV	=	Ultraviolet

CHAPTER 1

Introduction

Human JC virus (JCV) belongs to the family papovaviridae and subfamily polyomavirinae. The existence of polyoma viruses was first described in 1965 (Zu Rhein and Chou, 1965). The severe demyelinating brain disease called progressive multifocal leukoencephalopathy (PML) had been described previously (Astrom et al, 1958). A role of polyoma viruses in the etiology of PML was suspected, since particles resembling the polyoma virus virions were detected by electron microscopy in the brains of patients with PML (Zu Rhein and Chou, 1965; Silverman and Rubinstein, 1965). A polyoma virus was isolated from the brain of a PML patient. This virus was named JCV from the initials of the PML patient (Padgett et al, 1971). Further, another polyoma virus, BK virus (BKV), was isolated from the urine of a renal transplant patient (Gardner et al, 1971). It was thought initially that BKV and SV40, a virus introduced into many of the recipients of early polio vaccines due to contamination from monkey cells used in vaccine preparation, were also associated with PML (Weiner et al, 1972; Gribble et al, 1975; Holmberg et al, 1977; Scherneck et al, 1981). Current evidence indicates that JCV is the only or major viral cause of PML (Eizuru et al, 1993). Subsequent to the isolation of JCV in 1971, evidence that JCV is associated with PML was reported by several laboratories (Gardner,1977; Taguchi et al, 1982; Walker and Padgett, 1983).

1.1 SERO-EPIDEMIOLOGY OF JC VIRUS

Once the association of JCV with PML was established, serological surveys were used to gain insight into the incidence of the disease. Serological studies conducted in Wisconsin showed that 65% of individuals were positive for anti-JCV antibodies by the age of 14 (Padgett and Walker, 1973). The high prevalence of antibodies against JCV was further reported from different regions of the world.

Inapparent primary infection by JCV occurs in childhood and approximately 10% of children between the ages of 1 and 5 years demonstrate antibody to JCV. By 17 years of age, 65% of adults will have seroconversion and the adult population in some urban areas show as high as 92% seroconversion (Walker and Padgett, 1983). The virus is transported to the kidney and probably persists there indefinitely (Arthur et al, 1988 and 1989). In noncompromised individuals attending urologic clinics, JC viral DNA was detected in 29% and the presence of JCV DNA increased with age and peaked at 45% in the 60-89 years age group. Importantly, under immunocompromising conditions, such as renal and bone marrow transplantations, pregnancy, elderly and cancer, the virus is reactivated and can infect the brain (Hogan et al, 1980a, 1983; Gardner et al, 1984; Andrews et al, 1988; Arthur et al, 1988; Coleman et al, 1980 , 1983; Gibson et al, 1981; Daniel et al, 1981; Kitamura et al, 1990). It has been demonstrated that 55 to 85% of cases

of PML have AIDS, again supporting the theory that reactivation of JCV is associated with immunosuppression (Berger et al, 1987; Krupp et al, 1985). Recently, overwhelming evidence has been found for the association of PML with the AIDS epidemic and PML is a frequent complication of AIDS (Quinlivan et al, 1992).

Direct interaction of the AIDS virus (HIV type I) and JCV proteins was also reported (Gendelman et al, 1986; Tada et al, 1990). PML is the cause of death of 4% AIDS patients in North America (Markowitz et al, 1993). Both pregnant women and immunosuppressed patients who do not have PML shed JCV in their urine, suggesting that loss of immunocompetence predisposes individuals to the reactivation of a latent infection (Coleman et al, 1980; Gardner et al, 1984). However, the exact mechanism of primary infection by JCV has remained unclear.

1.2. MODE OF TRANSMISSION OF JCV

As JCV incidence is frequent in pregnant women, studies on the congenital transmission of the virus were attempted. Sera from the umbilical cord of infants of normal mothers did not reveal any JCV-specific IgM antibodies (Gibson et al, 1981) and fetal tissues also failed to show the presence of virus (Andrews et al, 1983). Serological studies confirmed that humans are the natural reservoir of JCV and that the virus is not transmitted by animal intermediates (Padgett et

al, 1977).

1.3. JC VIRUS GENOME

1.3.1. GENERAL FEATURES

The genome of prototype strain, MAD1 contains a 5130 bp covalently closed circular double-stranded DNA genome. The genome of this virus encodes two non-structural proteins, large T and small t antigens, three structural proteins named VP1, VP2, VP3 and an agnoprotein (Fig.1.1; Frisque et al, 1984). JCV exhibits close sequence homology to two other well characterized primate papovaviruses, the simian virus (SV40), and the human BK virus (BKV) (Osborn et al, 1974; Frisque et al, 1984). JCV and BKV share approximately 80% homology between their early proteins and late viral capsid proteins. JCV and SV40 share approximately 70% homology in these regions. However, unlike BKV and SV40, JCV has a narrow tissue tropism and JCV replicates efficiently exclusively in primary human fetal glial cells rich in spongioblasts, the precursors of oligodendrocytes (Aksamit and Proper, 1988; Wroblewska et al, 1980). In transgenic mice expressing JCV T-antigen JCV induces dysmyelination in central nervous system but not peripheral nervous system (Small et al, 1986). Subsequent to the isolation of the JCV for the first time in 1971, new isolates were reported. The first isolates of JC virus were reported from the University of Wisconsin in Madison and the isolates were designated as Mad1 (the prototype), Mad2, etc.

The prototype strain, Mad1 , contains two 98 bp repeats with one TATA box in each as part of its regulatory region (Martin et al, 1985; Loeber and Dorries, 1988). Most of the isolates of JCV were from the brain and few were from kidneys, urine of PML patients, renal and bone marrow transplant recipients or from normal individuals. The genome of JCV isolated from the urine of non-PML elderly individual who had no signs of immunosuppression contained a 23 bp insertion which was not found in prototype Mad1 strain. A 66 bp insertion was also found in the genome between nt 80 and 98 of the tandem repeat. The JCV genome with sequence rearrangements that includes 23 and 66 bp insertion is called the "archetype" genome (Yogo et al, 1990).

1.3.2. REGULATORY REGION OF JCV:

The sequences corresponding to nucleotide positions 5112 to 270 form the non-coding region of JCV (Fig. 1.1). It consists of promoter-enhancer elements essential for transcription and also the core sequences important for viral DNA replication. The regulatory region of JCV is functional in both directions. In one direction, the early region encodes large T and small t antigens. Second expression from the late promoter-enhancer leads to the formation of structural proteins of the virus. The sequences of replication origin of SV40, BKV, and JCV are well conserved, whereas, the sequences of promoter-enhancer elements diverge greatly. The regulatory

region of JCV contains two 98 bp tandem repeats (Fig. 1.1) and the unique feature of this virus, compared with BK and SV40 viruses, is that the TATA box is duplicated in each of the 98 bp repeats. The regulatory region also contains several cis-acting elements bound by cellular transcription factors.

The activity of JCV regulatory region unlike those of SV40 and BKV, is strictly restricted to glial cells. It has been suggested that the JCV control region consisting of the promoter-enhancer elements is responsible for the restricted tissue specificity, because the greatest degree of divergence is noticed in the regulatory region of JCV compared to those papovaviruses such as BKV and SV40 (Kenney et al, 1984; Nakshatri et al, 1990a).

1.3.3. NON-STRUCTURAL PROTEINS:

1.3.3.1. LARGE T- ANTIGEN:

The large T-antigen of JCV has not been studied in as much detail as the SV40 T-antigen. The large T-antigen of SV40 exhibits several properties like specific and non-specific DNA binding, alpha polymerase-primase binding, p53 and Rb binding, ATPase and helicase. These features have been correlated with SV40 transforming and oncogenic properties (Fanning, 1992). Since JCV and SV40 large T-antigens bear significant homologies, it was predicted that JCV T-antigen exhibits most of the properties that SV40 T-antigen does. Consistently, studies with JCV T-antigen showed that it has DNA (Lynch and

Frisque, 1991), p53 (Bollag et al, 1989; Haggerty et al,1989), and Rb (Dyson et al, 1989; 1990) binding activities. It has transforming (Frisque et al, 1980) and transregulating (Tada et al, 1990) properties. In addition, it mediates DNA replication of the JCV (Sock et al, 1991; Lynch and Frisque 1991).

1.3.3.2. SMALL t-ANTIGEN:

The JCV early RNA transcript is differentially spliced to produce small tumor (t) RNA (Fig.1.1). The large T and small t-antigens have the same amino terminal region but differ in their carboxyl terminal regions (Frisque et al, 1984). Like large T-antigen, the small t-antigen of JCV is not studied in detail. However, based on the sequence homology (82%), it is presumed that JCV and SV40 small t-antigens behave identically. SV40 small t-antigen was found to be necessary for complete transformation of non-permissive cells (Bouck et al, 1978; Sleigh et al, 1978). Small t-antigen also enhances the transactivating function of large T-antigen (Bikel et al, 1987).

1.3.4. STRUCTURAL PROTEINS:

1.3.4.1. CAPSID PROTEINS:

Due to the difficulty in propagating JC virus in cell culture, limited work has been done on the structural proteins. The largest open reading frame located near the 3'end of the late region encodes the 354 amino acid VP1 capsid

protein, the major structural protein. It plays an important role in the cell adsorption. The smallest open reading frame at the 5′ end of the late region encodes a 71 amino acid polypeptide, the agnoprotein. The exact function of this protein is not clear but it is assumed that it binds DNA and is involved in the viral maturation pathway. Between the 5′ and 3′ open reading frames of agnoprotein and VP1 proteins are two open reading frames that encode VP2 and VP3 capsid proteins of 344 and 225 amino acids, respectively (Frisque et al, 1984; Fig. 1.1).

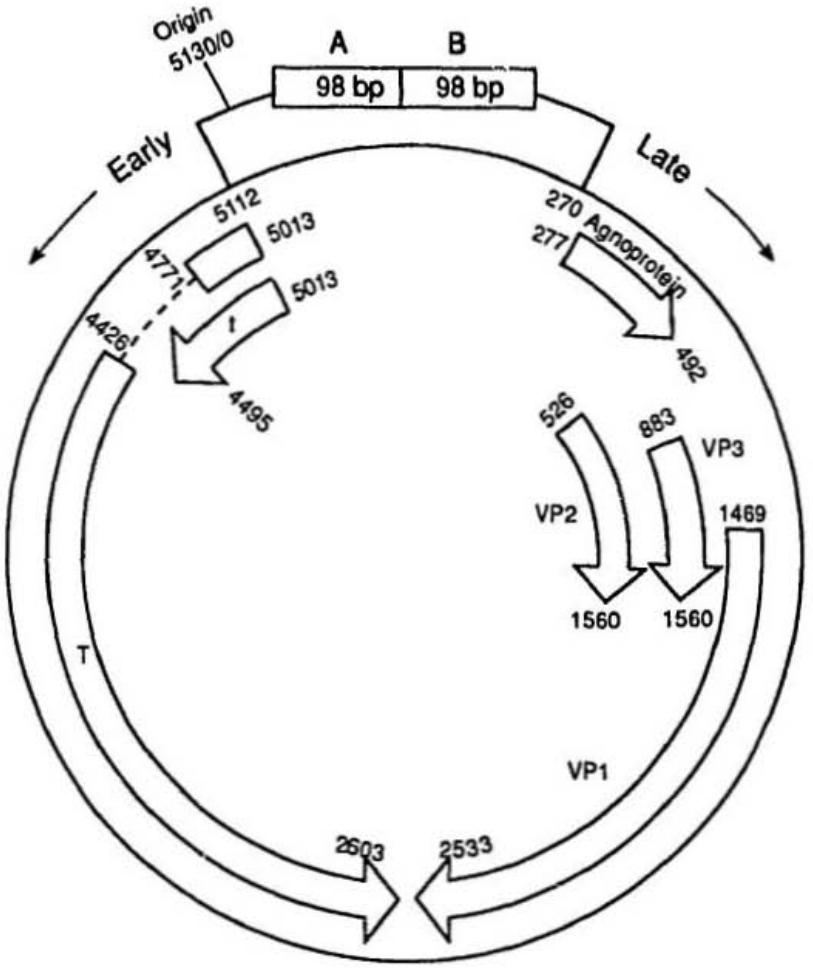
1.4. HETEROGENEITY OF JCV REGULATORY REGION

JCV DNA isolated from infected glial cells is variable in size and considerably shorter than tne full length infectious genome (Osborn et al, 1974; Howley et al, 1976; Martin et al, 1979; Grinnel et al, 1983). The multiplicity of infection (moi) and the cell population influence the degree of heterogeneity of JCV genome. For example, when passaged in oligodendrocyte rich cultures of primary human fetal glial(PHFG) cells at low moi, JCV DNA of unit length were observed. However, when passaged in astrocyte rich cultures with high moi, heterogeneous populations of JCV DNA were recovered (Martin et al, 1983). This suggests that the heterogeneity of the JCV genome depends on the in vitro propagation conditions, the source of the DNA and the natural genetic variation of the isolate.

Fig. 1.1 Circular diagram of the JCV genome.

The early and late regions are shown in the anticlockwise and clockwise directions, respectively. The numbers indicate nucleotide (nt) positions. The relative positions of the origin of DNA replication and the 98 bp repeats in the regulatory region of the JCV are indicated on the top. The open reading frames are shown as large open arrows of early proteins, T-antigen (T) and t. The dashed lines indicate intron sequences in large T-antigen coding sequences. The four open reading frames of late structural proteins of the virus are shown on the right side.

*This figure is made based on the Fig. 3 of Major et al (1992)


Origin
5130/0
A
B
98 bp
98 bp
Early
Late
5112
5013
4771
5013
t
4426
4495
270 Agnoprotein
277
492
526
883
VP3
VP2
1469
1560
1560
T
VP1
2603
2533

Initial studies were focused on the analysis of JCV DNA from brains and kidneys of PML patients. The two types of DNA varied in size and restriction patterns (Dorries 1984). Subsequently, a total of 13 DNA isolates from 6 different patients were cloned from brain tissue or virus that had been propagated in cell culture (Martin et al, 1985). The results suggested the presence of two types of JCV genome, type I and type II. The regulatory region of type I isolates were identical and contained the two 98 bp tandem repeats with duplicated TATA box, one in each of the repeats, lacked the consensus Sp1 binding site and thus resembled the prototype MAD1 strain. Type II genomes differed from one another but had several features in common. Each of them had lost one TATA box and diverged from the Mad1 sequence at nt 36. In addition, type II genomes had a 23 bp insertion which includes Sp1 recognition site present in the archetype sequences. Several isolates, namely Mad4, Mad8-Br, Mad11-Br and Her1-Br were recovered during isolation studies. All of these genomes are of type II except Mad1 and Mad4. Mad4 strain resembles Mad1 except for the absence of distal TATA box. The other variants are all of type II, having 23 bp insertion with Sp1 recognition site. Mad11-Br contains the 66 bp and 23 bp insertions (Matsuda et al, 1987; Major et al, 1987; Yogo et al, 1990).

Interestingly, passage of the virus from the infected brain tissue in PHFG cells resulted in a number of rearranged regulatory regions. Some of these had lost the 23 bp sequence found in type II regulatory regions, and thus resembled the Mad4 isolate. In order to clearly understand the differences in the regulatory regions of JCV genomes isolated from kidney and brain of PML patients, sequencing of the JCV DNA isolated directly from kidney and brain tissue of a PML patient was attempted. The brain isolate designated as GS/B was slightly larger than the kidney isolate, GS/K. Comparison of coding regions of the Mad1 and GS/B DNAs revealed a total of 96 nt differences and only 4 amino acid changes were detected in the large T-antigen coding region. Major sequence differences were noticed in the regulatory regions of Mad1, GS/B, and GS/K genomes (Loeber and Dorries, 1988).

The regulatory regions of Mad1 and GS/B contained two 98 bp repeats and two repeats of 63 and 93 bp, respectively. Sequences from 63 bp and 5′ end of 93 bp repeat of GS/B were highly homologous to the 3′ region of 98 bp repeat of Mad1. The GS/B sequences resembled that of Mad1 strain to a certain extent and also contained tandem repeats. The sequence of GS/K strain differed from GS/B by not having large tandem repeats and also by containing Sp1 factor binding site and 23 bp insertion, which was highly suggestive of the presence of archetype sequences. Based on these results, it was thought

that the hypervariability of JCV regulatory sequences may be a process of adaptation of JC virus from the kidney to the brain prior to the onset of PML (Loeber and Dorries, 1988).

Subsequent attempts were made to isolate JCV directly from non-PML patients to find out whether the kidney form of JCV differs from that of isolates from PML patients. Successful attempts in this direction have been reported (Yogo et al, 1990). JCV DNA from the urine of 8 non-immunosuppressed patients and two healthy individuals was analyzed in Japan. The sequences of all the clones were related to each other and differed only at 4 nt positions. The genomes resembled archetype DNA with one TATA box and a 23 bp insertion relative to the MAD-types. Interestingly, the sequences of these isolates from Japan were similar to the GS\K strain isolated in Germany several years earlier, except for the difference in 3 nt positions. These results suggested that the JCV DNA from the urine of healthy and nonimmunocompromised individuals predominantly contains archetype DNA. Hence, it was concluded that the brain-specific forms are generated from archetype sequences by DNA rearrangements.

1.5 LATENCY OF JCV

A longstanding puzzle has been the cell type in which JCV is latent. The brains from immunosuppressed and nonimmunosuppressed patients without PML contained no JCV early and late proteins by immunocytochemistry and the JCV

genome could not be detected by in situ hybridization (Hogan et al, 1980b). Recently, PCR technology has been used to demonstrate the presence of viral DNA in brain tissue from PML patients (Arthur et al, 1989; Telenti et al, 1990). However, PCR analysis did not detect JCV DNA in healthy brain tissue or brain tissue with other neurological abnormalities (Henson et al, 1991). Taken together, these observations suggest that JCV remains latent outside the central nervous system (CNS) and then gains access to the nervous system during immunosuppression. JCV is shed in the urine of pregnant women and immunosuppressed renal transplant patients. This indicates that the virus is latent in genitourinary tract (Coleman et al, 1980). In one study it was found that 10% of healthy individuals have integrated JCV DNA in renal tissue (Chesters et al, 1983). Using dot blot hybridization, JCV genome was detected in the kidney of five of seven PML patients (Grinnel et al, 1983).

Presence of archetype DNA is also demonstrated in normal renal tissues (Tominaga et al, 1992., White et al, 1992). The phylogenetic analysis indicated that the archetypal regulatory sequences are conserved in the course of JCV evolution (Iida et al, 1993). These observations further supported the previous assumptions that the JCV is latent in the kidney and during the immunosuppression, the regulatory region undergoes rearrangements and subsequently infects the

brain. Recent findings on archetype JCV DNA provided evidence for the generation of a brain-specific form from the archetype. The sequences corresponding to nt 177-204 and 192-219 in archetype JCV contain high homology to V-(D)-J recombination signals. A host cell DNA-cutting or recombining activity might target the viral regulatory region leading to its rearrangements. Such sites of breakage and rejoining are present only at the sites mentioned above. The rearrangements in the archetype JCV DNA sequences ultimately lead to the generation of infectious brain-specific form (Ault and Stoner, 1993). Support for this comes from another recent study which showed that the incidence of rearranged JCV regulatory regions compared to archetypal sequences is higher in immunosuppressed renal transplant recipients than in nonimmunosuppressed patients (Kitamura et al, 1994).

JCV DNA sequences have also been detected in B-lymphocytes of normal individuals and PML patients (Tornatore et al, 1992). Using in situ hybridization, presence of JC virus DNA was demonstrated in mononuclear cells at 200-1000 copies per cell, indicating the replication of viral genome (Houff et al, 1988). The B-lymphocytes also possess transcription factors that bind to the regulatory region of JCV (Major et al, 1990). Taken together, these observations suggested a model wherein the JCV can be present in the bone marrow during latency and subsequently, the virus infected

lymphocytes enter the perivascular space of the brain resulting in infection of the brain. It has been demonstrated recently that the nuclei of peripheral blood leucocytes of immunocompetent individuals harbour JC virus thus providing a strong evidence for the persistence of JCV in leucocytes of healthy individuals. In addition to kidney and CNS, the leucocytes are also targeted by JCV and upon immunosuppression the virus is reactivated and can infect the brain (Dorries et al, 1994).

1.6. RESTRICTION OF JCV GROWTH TO GLIAL CELLS IN CELL CULTURE

The major difficulty in studying JCV is the identification of a suitable cell culture system that allows its growth. Human embryonic cells from the kidney, lung, liver, amnion, and intestines were tested for expression of JCV (Beckman et al, 1982; Beckman and Shah, 1983; Miyamura et al, 1985). After several weeks in culture, only a few T-antigen positive cells were detected in human embryonic kidney (HEK) cells while the other cell systems were found negative. Later on, monkey kidney cell lines such as, BSC-1, CV-1, and Vero cells that supported the growth of SV40 virus were tried and were not found useful.

These observations raised the question of whether the observed host cell restriction was at the level of virion adsorption or penetration. To address this question, several JCV isolates were used to infect HEK and human lung cells by

transfecting their DNAs. In these studies, primary human fetal glial (PHFG) cells were used as positive control. Only DNA from one JCV MAD type produced T-antigen and late capsid proteins in all the cells tested. DNA of other types were infectious only for glial cells. Therefore, it was concluded that the restricted cell-specificity was not at the level of virion adsorption or penetration but could be due to intracellular factors that control the early gene expression and replication (Frisque et al, 1979).

Consequently, primary human fetal glial (PHFG) cells proved to be the best host for JCV growth. The propagation of JCV was enhanced by elevating incubation temperature from 37^0 C to 39^0 C (Grinnel et al, 1982). Cultures that are rich in spongioblasts, the precursor of the oligodendrocytes supported optimum growth of JCV (Padgett et al, 1977). A large number of virions were detected under the electron microscope in the nuclei of spongioblasts and rarely in astrocytes, one of the types of glial cells. However, efficient growth of JCV in astrocytes was reported in another study (Major and Vacante, 1989). No major alterations in the genome, transcriptional activity, virus titres, and lytic cycle were observed in spongioblast rich cultures even after continuous passage. Although the PHFG cells provided a better environment for JCV growth, it is difficult to cultivate these cells. Availability of these cells is another limiting factor. In addition, these

cells contain heterogeneous population of cells and most studies showed that spongioblasts permit maximum yields of virus.

Attempts were then made to look for a permissive cell system that does not have the disadvantages mentioned above. Serial passages of JCV Mad1 strain in HEK cells resulted in an adapted virus namely, JCV-HEK. This new adapted virus grew efficiently in kidney cells because of deletions and rearrangements in the regulatory region (Yoshiike et al, 1982; Miyamura et al ,1985). Attempts to grow the virus in cells from adult brain were also unsuccessful (Wroblewska et al, 1980). To eliminate all the above mentioned disadvantages, a human fetal glial cell line was developed by immortalization of astrocyte cells with a replication defective SV40 DNA clone that expresses high levels of large T-antigen (Major et al, 1985). This cell line was susceptible to JCV infection. Immortalization of oligodendrocyte precursor cells that divide poorly in the culture was unsuccessful. PHFG cells were transformed by origin defective JC virus to generate a cell line called POJ that allowed maximum growth of JCV (Mandl et al, 1987). Recently, productive infection of JCV was also reported in Schwann cells, the cell type responsible for myelin production in the peripheral nervous system (Assouline and Major, 1991). Overall, the growth and expression of JCV is essentially restricted to glial cells only.

1.7 TRANSCRIPTION

The restricted cell specificity of JCV is largely due to its control region. The activity of JCV regulatory region is limited to glial cells, unlike those of BK and SV40 viruses. Hence further studies were attempted to find out the promoter-enhancer elements and cellular transcription factors that contribute to glial cell specificity of JCV. Before explaining the transcriptional regulation of glial cell-specific expression of JCV, I would like to describe the general transcription machinery and how cell-specific gene expression is controlled at the level of transcription.

1.7.1 PROMOTER AND ENHANCER ELEMENTS

In eucaryotes, promoters transcribed by RNA polymerase II consists of two elements: the TATA box which is usually located 30 nt upstream of the transcription start site, and the initiator elements which are at the start site. These sequences are called the minimal or core promoter elements (Smale and Baltimore, 1989). The TATA box is essential for determining the transcription start site. In promoters lacking TATA box, for example in case of terminal deoxynucleotidyl transferase gene, the initiator element specifies the transcription start site.

The second class of elements called "enhancers" or "upstream promoter elements (UPE)" are recognised by sequence-specific DNA binding proteins that activate transcription from

the promoter. The enhancers are of two types: inducible and tissue specific enhancers. The enhancers which are responsive to stimuli such as hormones, heat shock, metals, and growth factors are called inducible enhancers. Examples include metallothionein, β-interferon, c-fos, and glucocorticoid hormone response element (Maniatis et al, 1987; Mitchell and Tjian, 1989). The enhancers which are active in certain stages of embryonic development and in specific tissues are called tissue-specific enhancers. The examples for this class include B-cell specific immunoglobulin enhancers, liver cell-specific aminotransferase gene, and brain-specific myelin basic protein gene (Maniatis et al, 1987).

1.7.2 TRANSCRIPTION FACTORS

1.7.2.1 GENERAL TRANSCRIPTION FACTORS

The general transcription factors (GTFs) which are important for basal transcription from the promoter are TFIID, TFIIA, TFIIB, TFIIE, TFIIF, TFIIH, TFIII, and TFIIJ. Most are multimeric complexes which include polypeptides with multiple functional and structural domains (Swadogo and Sentenac, 1990; Zawel and Reinberg, 1993).

TFIID is a multisubunit complex consisting of TATA box binding protein (TBP) and the proteins associated with TBP called TBP associated factors (TAFs) (Pugh and Tjian, 1990). TBP, a 38 kDa protein, specifically recognises and binds the TATA box and is important for transcription initiation (Hawley

and Roeder, 1985; Swadogo and Roeder, 1985). The carboxyl terminus of TBP is highly conserved in a variety of species and the amino terminus is highly divergent (Hoffman et al, 1990). The carboxyl terminal domain is essential for DNA binding and species specificity (Cormack et al, 1991; Reddy and Hahn, 1991). TFIID binds in the minor groove and induces bending of DNA. This facilitates the interaction of TFIID with other transcription factors and TAFs (Horikoshi et al, 1992; Starr and Hawley, 1991). Recently, it has been shown that the promoters transcribed by all three polymerases named polymerase I, II, and III, including TATA-less promoters, require binding of TBP (Hernandez, 1993; Zhou et al, 1992). The three dimensional TBP structure revealed the saddle shaped DNA binding domain. The concave surface of the saddle makes the specific and non-specific contacts with DNA. The convex surface is made available to the interaction with TAFs, GTFs and gene specific regulators (Kim et al, 1993a; Kim et al, 1993b; Nikolov et al, 1992).

TFIIA stabilizes the TFIID/DNA interactions. In vitro, TFIIA stimulates basal transcription when native partially purified TFIID is used but not when recombinant TBP is used. This indicates that TFIIA is essential to remove a component that negatively regulates TFIID activity (Reinberg et al, 1987). TFIIA is a complex of 35, 19, and 12 kDa subunits (Cortes et al, 1992; Coulombe et al, 1992a). A cDNA, hTFIIA/α

encodes both the p35 and p19 subunits of human TFIIA. It was suggested that a protease specifically interacts with the hTFIIA and cleaves 55kDa precursor protein into p35 and p19 subunits (De Jong and Roeder, 1993; Ma et al, 1993; Yokomori et al, 1993a). It has been shown recently that TFIIA has no role in basal transcription. Instead, it acts as a coactivator in activating transcription by gene specific activators by competing with negative regulators of TBP (Ma et al, 1993).

TFIIB interacts with DA complex through the sequences of the carboxyl terminus. The TFIIB amphipathic alpha helical amino terminus is proposed to interact with transcriptional activators (Ha et al, 1991).

TFIIE consists of two subunits, 57 kDa IIE α and 34 kDa IIE β. Both subunits contain sequences homologous to bacterial sigma factor (Ohkuma et al, 1991; Sumimoto et al, 1991). TFIIE stimulates the TFIIH-dependent kinase activity that phosphorylates the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II (Lu et al, 1992).

TFIIF consists of two subunits of 30 and 74 kDa. Hence it is also called RAP30/74. TFIIF interacts with RNA polymerase II and recruits polymerase to the promoter by preventing interaction with non-specific sites. Since TFIIF exhibits ATP dependent DNA helicase activity, it may play a role in the unwinding of the promoter DNA to form an open complex (Aso et al, 1992; Greenblatt, 1991; Finkelstein et al,

1992).

TFIIH contains seven subunits with molecular masses of 94, 75, 62, 46, 43, 38, and 35 kDa (Ohkuma and Roeder, 1994). Hydrolysis of the β-γ bond of ATP is essential for transcription initiation and part of the energy is used for unwinding the DNA double helix in the region of the initiation site (Swadogo and Roeder, 1984). TFIIH possesses DNA-dependent ATPase and kinase activities. It phosphorylates CTD of RNA pol II (Lu et al, 1992). Recently, it has been demonstrated that in vitro in the absence of TFIIE, TFIIH can not act on CTD because of the tight association of CTD with TBP and DNA template. Addition of TFIIE enables TFIIH to enter the preinitiation complex, thereby resulting in phosphorylation of CTD of pol II, melting of DNA around start site. This finally leads to transition from transcription initiation to elongation (Ohkuma and Roeder, 1994). The cloning of one of the subunits of TFIIH is an exciting development in understanding the possible roles of GTFs. It has sequence homology to ERCC-3 gene product, a helicase involved in human DNA excision repair disorders such as xeroderma pigmentosum and Cockayne's syndrome (Schaeffer et al, 1993; Sung et al, 1993). TFIIJ is essential for transcription when highly purified TFIID is used but not crude TFIID.

The ordered assembly of these GTFs at the promoter region specified by TATA box is a prerequisite for the

transcription initiation by polymerase II leading to basal level of transcription (Flores et al, 1992). There are also complex mixtures of poorly understood polypeptides interacting at the initiator (Inr) elements (Zawel and Reinberg, 1993).

1.7.2.2 TRANSCRIPTION ACTIVATORS

Another class of transcription factors are called activators. Activators and their DNA recognition sequences form a major focus of my studies. Activators interact with the enhancer sequences, are modular and contain two domains: DNA binding and activation domains. DNA binding domains include zinc finger, leucine zipper, homeodomain and helix-loop-helix motifs. The activation domains are of three kinds, acid rich, proline rich, and glutamine rich (Mitchell and Tjian, 1989). Transcriptional activators stimulate the rate of transcription by contacting one or more of the general transcription factors assembled at the promoter (Ptashne and Gann, 1990).

1.7.2.2.1 MECHANISM OF TRANSCRIPTIONAL ACTIVATION BY ACTIVATORS.

In general, eucaryotic gene expression requires transcriptional activators binding to cis-acting elements located either upstream or downstream of the transcription start site (Mitchell and Tjian, 1989; Ptashne, 1988; Struhl, 1989). The cis-acting elements are specific to each gene and are recognised by ubiquitous, development-stage-specific, or tissue-specific DNA binding proteins interacting with the

basic transcriptional machinery via their transcriptional activation or inhibition domains (Swadogo and Sentenac,1990). The interaction may depend on DNA looping or other chromatin structures (Ptashne, 1988). The greater details of the mechanism are discussed in the following subsections.

1.7.2.2.1.1 INTERACTION OF ACTIVATORS WITH GENERAL TRANSCRIPTION FACTORS (GTFs)

TFIID is essential for the assembly of the pre initiation complex (PIC) on all promoters tested in vitro. Thus TFIID was thought to be a target for certain activators. The mammalian transcription activator, activating transcription factor (ATF), stimulated the rate of transcription from adenovirus E4 promoter by interacting with TFIID (Horikoshi et al, 1988a). Subsequently, TFIID was shown to be a target for activators like Gal4 (Horikoshi et al, 1988b) and upstream stimulatory factor (USF) that activates adenovirus major late promoter (Swadogo and Roeder 1985). The pseudorabies virus immediate early protein (IE) stimulates transcription in vitro by accelerating the recruitment of TFIID to TATA box (Abmayr et al, 1988). Adenovirus E1A protein, a potent transactivator of transcription was shown to interact with carboxyl terminal basic repeats of TFIID in order to generate a rapid, efficient assembly of PIC (Lee et al, 1991).

Generally, other viral transactivators like herpes simplex VP16 (Stringer et al, 1990) and papillomavirus E2 protein (Dostani et al, 1991) enhance the rate of transcription by interacting with TFIID and stabilizing the complex at the promoter. Epstein-Barr virus Zta protein, which contains a transcription activation domain different from E1A, interacts with carboxyl terminus of TFIID and stabilizes the association of TFIID with the promoter (Liebermann and Berk, 1991). The activator proteins such as p53 (Truant et al, 1993) and PU.1 (Hagemeier et al, 1993) have been shown to directly interact with TBP. The tat protein of HIV transactivates transcription in vivo and in vitro. Direct interaction of tat with TFIID composed of TBP and associated factors (TAFs) was observed. This suggested that tat may transduce regulatory signals by interacting with TFIID (Kashanchi et al, 1994).

The chimeric activator termed Gal4-VP16 consisting of Gal4 DNA binding domain fused to acidic rich transactivation domain of VP16 stimulates transcription by recruiting TFIIB to the pre-initiation complex (Lin and Green, 1991). LSF cellular factor accelerates the association of the TFIIB with TATA bound TFIID and thereby stimulates the rate of transcription from SV40 major late promoter (Sundseth and Hansen, 1992). These examples demonstrate that activators accelerate the rate of transcription by facilitating the recruitment of GTFs to promoter.

1.7.2.2.1.2 INTERACTION WITH TBP ASSOCIATED FACTORS (TAFs)/COACTIVATORS

Initial studies with purified TBP exhibited only basal but not activated levels of transcription of core promoters in vitro even in the presence of transcriptional activators like Sp1, USF, and NF1. Instead, partially purified TFIID allowed transcriptional activation by activators in vitro suggesting that the proteins associated with TBP called TAFs are important for activated transcription. Some of the TAFs can be dissociated from TBP under conditions of high ionic strength and some are tightly associated with TBP and require denaturing agents like urea for removal from TBP. This suggests that some TAFs are always associated with TBP and some interact with TBP transiently (Drapkin et al, 1993).

Subsequently TAFs were isolated from crude TFIID using denaturing agents like urea. Addition of these TAFs to the recombinant TBP stimulated the rate of transcription in the presence of activator proteins such as, Sp1 and CTF/NF1 in vitro (Tanese et al,1991). Thereby, it was suggested that the activators interact with one or more of these TAFs directly and regulate the activity of TBP by altering DNA-TBP interactions. The components of TAFs that interact with activators and form a bridge between TBP and activators to stimulate the rate of transcription from the core promoters are also called co-activators.

At present 13 TAFs have been isolated that mediate activator-dependent transcription by RNA polymerase II. Two complexes containing TBP were isolated from HeLa cells. One of the complexes isolated from the 0.85 M KCl fraction contained 13 TAFs with molecular weights 230, 135, 115, 100, 95, 80, 55, 31, 30, 28, 20, 19, and 18 kDa and supported activated levels of transcription by RNA pol II in the presence of activator proteins. The other complex isolated from 0.3 M KCl fraction contained 4 TAFs of 190, 96, 87 and 60 kDa that substituted for TFIIIB factor required for RNA pol III transcription (Chiang et al, 1993). The pol I transcription factor SL1 contains three TAFs with molecular weights 110, 63, and 48 kDa which are distinct from TAFs important for pol II and III transcription (Comai et al, 1992). This suggests that there are Pol I, II, and III specific TAFs mediating activator-dependent transcription.

Not all these TAFs have been cloned. The TAF 250 (Hisatake et al, 1993; Kokubo et al, 1993; Ruppert et al, 1993) and Drosophila TAFs 110 (Hoey et al, 1993), 80 (Dynlacht et al, 1993), and 40 (Goodrich et al, 1993) have been cloned. The human TAF 250 was identical to cell cycle regulator CCG1 and hence the suggestion was given that hTAF250/CCG1 may be involved in regulation of cell cycle progression (Hisatake et al, 1993). It has been shown recently that the $TAF_{II}250$ subunit of the TFIID rescues a temperature sensitive hamster cell line

to overcome a G_1 arrest. The nuclear extract from this cell line failed to support the activated levels of transcription by gene specific regulators, Sp1 and Gal4-VP16, without affecting the basal level of expression. This is the first report to demonstrate that a general transcription factor, TAF-TBP complex, influences the expression of genes responsible for cell cycle progression (Wang and Tjian, 1994). The Drosophila $TAF_{II}110$ does not bind TBP directly but interacts with $dTAF_{II}250$ and $dTAF_{II}30\alpha$ which bind TBP directly. TAF110 is thereby recruited to TBP (Kokubo et al, 1993; Yokomori et al, 1993b). In addition, $dTAF_{II}110$ also interacts with large subunit of TFIIA (Yokomori et al, 1993a).

The Drosophila TAF250 and 110 interact with Sp1 activator and stimulate the rate of transcription (Ruppert et al, 1993; Hoey et al, 1993; Weinzierl et al, 1993). Recently it has been shown that the glutamine rich hydrophobic surface of Sp1 interacts with $dTAF_{II}110$ and activates transcription (Gill et al, 1994). Another transcription factor CREB also interacts with $dTAF_{II}110$, apparently through the regions similar to that of Sp1. This suggests that different classes of activators utilize $dTAF_{II}110$ as a coactivator and stimulate the rate of transcription (Gill et al, 1994). However, in another study it was shown that the acidic activator containing an activation domain different from Sp1 and CREB interacts directly with another component of TFIID, $dTAF_{II}40$,

to activate transcription (Goodrich et al, 1993). This suggests that different classes of activators interact with distinct components of TFIID such as $TAF_{II}250$, 110, and 40.

The discovery of coactivators revolutionised the field of transcription, especially immunoglobulin gene transcription. The lymphoid cell-specific oct-2 protein but not ubiquitously expressed Oct-1 protein was shown to be important for B cell-mediated transcription in a number of studies (Tanaka and Herr, 1990; Gerster et al, 1990). Ig H promoter-specific, Oct-1 factor specific, and B cell-specific coactivator that stimulated Ig H promoter effectively in the presence of Oct-1 but not Oct-2 questioned the role of lymphoid cell-specific Oct-2 in immunoglobulin gene transcription (Luo et al, 1992). Further, the disruption of Oct-2 locus in B-cells had no effect on immunoglobulin gene transcription (Feldhaus et al, 1993), supporting the role of a B cell-specific coactivator in immunoglobulin gene transcription.

Dimerization of a liver specific transcription factor, Hepatocyte Nuclear Factor-1 (HNF1) is essential for the transcription of liver-specific genes. A liver-specific coactivator mediates the dimerization of HNF1 and thus plays a key role in liver-specific transcription (Mendel et al, 1991). The evidence for the role of coactivators in the transcription of eucaryotic genes, especially in tissue-

specific gene expression, is well documented.

A mediator activity distinct from TAFs was purified from yeast cells and shown to be necessary for stimulation by acidic activators such as VP16 and GCN4. The mediator facilitates direct interaction between activators and components of the basic transcription machinery (Kelleher et al, 1990; Flanagan et al, 1991).

1.7.2.2.1.3 STIMULATION OF BASAL TRANSCRIPTION BY RELIEVING REPRESSION

Recently, histone-mediated repression of transcription has received considerable attention. Order of addition experiments revealed that nucleosomes prevent pre-initiation complex formation by GTFs (Workman et al, 1991). Transcriptional activators like VP16 and Sp1 stimulated transcription of histone H1-mediated but not naked DNA templates by 200-fold by stabilizing and accelerating the recruitment of GTFs to the promoter (Croston et al, 1991; Laybourn and Kadonaga, 1991; Laybourn and Kadonaga, 1992).

An activity called upstream factor stimulatory activity (USA) distinct from co-activators was reported (Meisterernst et al, 1991). USA contains two components, negative cofactor 1 (NC1) and positive cofactor 1 (PC1). They exhibit opposite effects on core promoter activity. NC1 inhibits promoter activity by interacting with TBP, thus preventing interaction of TFIIA with TBP. On the other hand,

PC1 in combination with activators like Sp1 and USF reverse the action of NC1 by directly or indirectly facilitating the entry of TFIIA into pre-initiation complex (Meisterernst et al, 1991). In addition, other factors such as negative cofactor 2 (NC2) and TFIID binding factor 4 (DBF4) and with properties similar to NC1 were isolated that reduce the core promoter activity by forming a non-productive complex with TFIID. The transcriptional activators reversed the inhibition by facilitating the formation of a productive pre-initiation complex (Meisterernst and Roeder, 1991).

A novel repressor activity namely Dr1 was isolated from HeLa cell extracts that binds to TFIID and prevents the subsequent binding of TFIIA and B. However, activators like adenovirus E1A and SV40 large T-antigen disrupt this complex and facilitate the entry of TFIIA and TFIIB (Inostroza et al, 1992).

1.7.3. FACTORS THAT INFLUENCE THE TRANSCRIPTIONAL ACTIVATION BY GENE-SPECIFIC ACTIVATORS

1.7.3.1 DNA BINDING SITE AND TRANSCRIPTIONAL RESPONSE

The individual cis-acting element in the regulatory region of a gene can bind to one or many different factors which belong to the same or a different family. For example, many homeodomain proteins and ligand-dependent nuclear receptors bind to the same cis-acting element (Hoey and Levine, 1988; Glass et al 1988; Beato, 1989). A site in human

osteocalcin gene is recognised by both Jun/Fos and vitamin A and D receptors. However, each exerts an opposite transcriptional effect (Schulle et al,1990). Thus a single cis-acting element can elicit different transcriptional responses depending on the binding of the specific transcriptional factor.

On the other hand, a single transcription factor can bind to different DNA sequences. A good example for this is the yeast HAP1 zinc finger protein (Pfeifer et al, 1987). Other examples are certain homeodomain proteins (Hoey and Levine, 1988). Although different transcription factors bind the same cis-element, the relative position, orientation, and spacing of binding motifs can play an important role under certain conditions. For example, the glucocorticoid and the oestrogen receptors recognise related but distinct binding motifs (Klock et al, 1987). On the other hand, the receptors for oestrogen, thyroid hormone, retinoic acid, and vitamin D bind virtually identical sequences. However, the relative spacing and orientation determine the transcriptional response which can be either activation or repression (Glass et al, 1988; Naar et al, 1991; and Umesono et al, 1991).

1.7.3.2 INTRAFAMILY PROTEIN-PROTEIN INTERACTIONS

Some classes of transcription factors bind DNA as either homodimers or heterodimers. Jun and fos transcription factors belonging to the leucine zipper family bind DNA as

heterodimers to a cis-acting site called AP-1 site and some of them have different transcriptional activities (Nakabeppu et al, 1988; Cohen et al, 1989). The glucocorticoid and oestrogen receptors bind to their respective sites as homodimers (Kumar and Chambon, 1988; Tsai et al, 1988). The retinoic acid receptors bind DNA as heterodimeric complexes with other nuclear factors called coregulators, one of them being the thyroid hormone receptor (Glass et al, 1989, 1990). These coregulators affect DNA binding and transcriptional activity of retinoic acid receptor. The muscle specific helix-loop-helix protein, MyoD, dimerizes with ubiquitously expressed proteins of the same family to activate transcription of muscle-specific genes (Weintraub et al, 1991; Benezra et al, 1990).

Heterodimer formation sometimes results in inhibition of DNA binding and thereby represses transcriptional activity of activators. Id, a member of helix-loop-helix family, heterodimerizes with other members of the same family such as MyoD and E12/47 and inhibits the transcriptional activity of these factors (Benezra et al, 1990). I-POU, a protein belonging to POU domain class specifically heterodimerizes with another POU domain protein, cf1a, and prevents its DNA binding and transactivating neural specific genes (Treacy et al, 1991; Johnson and Hirsch, 1990).

1.7.3.3 INTERFAMILY PROTEIN-PROTEIN INTERACTIONS

Recently, it has been shown that glucocorticoids can inhibit the transcriptional activity of Jun/Fos on AP-1 site. Glucocorticoid receptor prevents binding of Jun/Fos complex to DNA. Also, the Jun/Fos complex prevents the glucocorticoid and other nuclear receptors from binding to their respective response elements (Schule et al, 1990; Jonat et al, 1990; Diamond et al, 1990). The VP16 transcription activator of herpes simplex virus cannot bind DNA alone, but interacts with the homeodomain protein Oct-1 through its acidic rich transcriptional activation domain to stimulate the rate of transcription (Stern et al, 1989; Kristie and Sharp, 1990). The pX protein of hepatitis B virus interacts with leucine zipper proteins CREB and ATF2 and enables them to bind to a different set of DNA sequences (Maguire et al, 1991).

1.7.3.4 COVALENT MODIFICATION OF TRANSCRIPTION FACTORS AND TRANSCRIPTIONAL ACTIVATION

Certain transcription factors require posttranslational modifications either to activate or inhibit transcription. The phosphorylation of the threonine residue within the DNA binding domain of Pit-1 pituitary specific transcription factor may differentially increase or decrease its binding affinity for the cis-acting element present in prolactin and growth hormone gene promoters (Kapiloff et al, 1991). The Jun transcription factor is phosphorylated near its N-terminal DNA

binding domain and protein kinase C activation leads to dephosphorylation at this site. This increases DNA binding and transactivation by the Jun/Fos complex (Boyle et al, 1991). Similarly, phosphorylation of serine residue by cAMP dependent protein kinase A is essential for transcriptional activation of cAMP response element binding protein (Gonzalez and Montminy, 1989; Sheng et al, 1990).

1.7.4 TRANSCRIPTIONAL REGULATION OF CELL-SPECIFIC GENE EXPRESSION

Cell-specific gene expression is mainly regulated at the level of transcription. The cis-acting elements and DNA binding proteins involved in the expression of various genes that are expressed in a cell-specific manner are well characterized, especially in the case of the liver (reviewed in Simone and Cortese, 1991; 1992; Lai and Darnell, Jr, 1991), muscle (reviewed in Olson, 1990; Tapscott and Weintraub, 1991; and Li and Olson, 1992), pituitary (reviewed in Sharp and Cao, 1990; Karin et al, 1990), erythroid (reviewed in Orkin, 1990) and immune systems (reviewed in Staudt and Lenardo, 1991).

1.7.4.1 MUSCLE-SPECIFIC GENE EXPRESSION

Muscle-specific expression is a well-studied system serving as a model of tissue-specific gene expression. Muscle-specific genes are mainly regulated at the level of transcription. Myoblast differentiation is accompanied by transcriptional induction of muscle-specific genes. Earlier

studies suggested the existence of a master gene important for setting the events that finally end up in the formation of mature fibre. The cDNA for the master gene, MyoD was cloned subsequently by subtraction hybridization using myoblasts and fibroblasts. Transfection of MyoD cDNA into 10T1/2 fibroblasts activated myogenesis (Davis et al, 1987). Subsequently, three more muscle specific transcription factors, myogenin (Edmondson and Olson, 1989), myf5 (Braun et al, 1989), and myf6 (Miner and Old, 1990; Braun et al, 1990) were isolated. Like MyoD, each of these factors can convert 10T1/2 fibroblasts to myoblasts.

MyoD alone can activate muscle specific gene expression or set the events that lead to activation of muscle specific genes. In cooperation with ubiquitously expressed factors MyoD can activate muscle cell-specific gene expression. The MyoD is a muscle cell-specific factor that binds to a CANNTG sequence called the "E-box site". The E-box binding site is present in many muscle specific genes such as muscle creatine kinase (MCK), troponin 1, alpha-cardiac actin and MLC1/3 genes. In addition, the E-box binding sites are also present in the regulatory regions of other cell-specific genes, such as immunoglobulin and insulin genes (Sen and Baltimore, 1986; Walker et al, 1990).

MyoD binds to DNA very weakly. However, in cooperation with the widely expressed E2A gene products such as E12, E47,

and TFE3 factors MyoD acquires high affinity for binding DNA and regulates transcription (Davis et al, 1990; Murre et al, 1989). Although the members of MyoD and E2A gene products binds to the same sequence, MyoD can activate the transcription of muscle cell-specific genes, whereas, E2A gene products can not (Blackwell and Weintraub, 1990). It was further shown that the basic region of MyoD plays a role in the transcriptional activation of muscle cell-specific genes (Davis et al, 1990).

Replacement of the basic region of MyoD with that of E2A gene products does not prevent the DNA binding activity of MyoD but fails to activate muscle cell-specific transcription. The mutations in the basic region of MyoD that allows DNA binding but prevent activation of muscle-specific genes was also reported (Davis et al, 1990). This observation provided a clue that the activation domain of MyoD is cryptic and the basic region somehow communicates with the activation domain to stimulate transcription upon binding to E-box. Three possible mechanisms were proposed. (1) The basic region may interact with a co-activator that results in communication of the basic region with the activation domain. (2) Both MyoD and myogenin may undergo a conformational change, unmasking the transcriptional activation domain. (3) Another mechanism may involve interaction of muscle specific factors with other transcription factors that bind to nearby sites in DNA leading

to activation of muscle cell-specific genes. The examples for this are the activation of MCK enhancer that requires cooperative binding of MyoD and other ubiquitous and muscle-specific transcription factors such as MEF2 (Weintraub et al, 1991). Also, the activation of human alpha cardiac actin promoter requires simultaneous binding of serum response factor, Sp1, and MyoD (Sartorelli et al, 1990).

1.7.4.2 BRAIN-SPECIFIC GENE EXPRESSION

JCV expression and replication is brain-specific. JCV is oncogenic in animals and it is the only human virus known to produce solid brain tumors in nonhuman primates (Frisque and white, 1992). Therefore, it is also important to understand brain-specific gene expression. The mammalian brain is a complex organ containing a heterogeneous population of cells. Hence it is very difficult to study its gene expression. Analogous to the cell-specific gene expression of liver, muscle and other organs, brain-specific genes are also regulated mainly at the level of transcription (reviewed in Takahashi, 1992; Mandel and McKinnon, 1993).

1.7.4.2.1 GLIAL CELL-SPECIFIC GENE EXPRESSION

JCV has similarities to the control regions of myelin basic protein (MBP), glial fibrillary acidic protein (GFAP), proteolipid protein (PLP), and S100β genes which are expressed mostly in glial cells as detailed in the following.

1.7.4.2.1.1 GLIAL CELL-SPECIFIC EXPRESSION OF GFAP

Three regions (A,B, and D) in the regulatory region of

GFAP were found important for its glial cell-specificity. The region A contains binding sites for transcription factors Sp1 and nuclear factor 1 (NF1). Region B contains adjacent activator protein 1 (AP1) and NF1 binding sites. Region D consists of adjacent NF1 and AP2 binding sites. Region B is the most active of the three regions and stimulates strong glial cell-specific expression. Regions A, B, and D activate heterologous promoters such as that of SV40, in glial cells but not in other cell types. Region D functions primarily to promote interactions that bring region B close to the promoter (Besnard et al, 1991).

1.7.4.2.1.2 EXPRESSION OF S100β IN GLIAL CELLS

S100β protein is primarily present in astrocytes and is found in 30 to 100 fold higher levels than in other tissues (Kato and Kimura, 1985). The binding sites for NF1, cAMP response element binding protein (CREB), and AP2 were found important for the expression of S100β in the glial cells (Allore et al, 1990).

1.7.4.2.1.3 GLIAL CELL-SPECIFIC EXPRESSION OF PLP

Proteolipid protein (PLP) is one of the components essential for the formation of myelin that forms a sheath around axons. The regulatory region of PLP contains NF1 binding sites and also both positive and negative cis-regulatory elements which determine the glial cell-specificity (Nave and Lemke, 1991). The cis-acting elements are highly

homologous to those observed in MBP.

1.7.4.2.1.4 MYELIN BASIC PROTEIN GENE EXPRESSION IN GLIAL CELLS

MBP forms one of the important constituents of myelin. MBP promoter consists of several regulatory sequences out of which the proximal element MB1 and one of the distal elements are highly active in glial cells as compared to other cells (Beach et al, 1990). The sequences around the NF1 binding sites in the proximal and distal regions were found to be important for glial cell-specific expression of MBP promoter (Aoyoma et al, 1990). Another surprising finding is that the TFIID from brain but not liver stimulated the transcription of MBP in vitro, suggesting that different tissues contain functionally different TFIID species (Tamura et al, 1990a). Hence the interaction of different proteins binding the MBP regulatory region may influence the glial cell-specificity.

1.7.4.2.2 NEURON-SPECIFIC GENE EXPRESSION

The genes for neurofilament, proenkephalin, and dopamine β-hydroxylase (DBH) genes are expressed only in the neurons of the central nervous system. The regulatory regions of neurofilament and proenkephalin contain adjacent NF1 and AP2 binding sites and cooperative interaction of these proteins may play a role in neuronal cell-specific expression (Comb et al, 1988; Nakahira et al, 1990).

A role for a negative regulatory element in the neuron specific expression of SCG10, a neuron-specific growth associated protein, and the type II sodium channel protein was recently reported. Deletion of the cis-acting negative regulatory element or silencer region allows expression of these genes in non-neuronal cells. Thus the silencer region plays a major role in the cell-specific expression of these neuronal genes (Mori et al, 1990; Maue et al, 1990). The sequences of the silencer region of these two unrelated neuronal genes is strikingly similar. In addition, the same protein from various non-neuronal cells was shown to interact with this silencer region (Mori et al, 1992; Kraner et al, 1992).

Dopamine β-hydroxylase, the enzyme catalyzing the conversion of dopamine to norepinephrine, is specifically expressed in adrenergic and noradrenergic neurons in the central nervous system. By DNase I hypersensitive assay and transient expression assays, a 4.3 Kb upstream sequence was found to be essential for neuronal cell-specific expression of DBH gene. By deletion and mutation analyses two regions in the regulatory region of DBH gene were identified that confer cell-specificity. The first region, the cAMP response element (CRE) is essential for positive regulation of DBH and confers 95% of cell specificity. The second region is a silencer region that negatively regulates its expression in non-

neuronal cells. Hence interplay between the CRE and neuron-specific silencer plays an important role in the tissue-specific expression of the DBH gene (Ishiguro et al, 1993). The tyrosine hydroxylase (TH) gene contains the consensus CRE in the proximal region and the CRE plays an important role in both the basal and cAMP inducible expression (Kim et al, 1993). Thus these two genes for enzymes TH and DBH adopt similar molecular strategies for their neuronal cell-specific expression by using same cis-acting element, CRE. As will be discussed later (chapter 5), JCV also contains adjacent Nuclear Factor 1 binding and CRE motifs (Amemiya et al, 1992).

1.7.5 GLIAL CELL-SPECIFIC EXPRESSION OF JC VIRUS

1.7.5.1 TRANSCRIPTION INITIATION OF JCV EARLY AND LATE GENES

The promoter-enhancer sequences of JCV were first identified based on the sequence comparison with BK and SV40 viruses. The peculiarity of JCV is that it contains two TATA boxes, one in each of the 98 bp repeats, in contrast to presence of single TATA box in SV40 virus (Frisque et al, 1984; Fig. 1.1). The presence of two TATA boxes in JCV promoter-enhancer was thought to control the early and late transcription of the virus. The viral early gene transcription was shown to initiate from nt 122-125 using TATA box located in the repeat B (located far from the origin of replication Fig. 1.1), as shown by S1 nuclease analysis (Kenney et al, 1986a). Using primer extension technique it was shown that the

early transcription initiated from nt 5112 and 5082 and the TATA box located in the repeat A (located near the origin of replication Fig. 1.1) and that repeat B was not utilized (Khalili et al, 1987).

Another study used primer extension technique to map the early transcription start sites to nt 5112, 5119, and 5090, analogous with the above study (Vacante et al, 1989). Early and late gene transcription was shown to utilize the TATA boxes located in the repeat A and B, respectively (Tada and Khalili, 1992). The discrepancies in the study of transcription start site have been resolved in a recent study. Using both primer extension and S1 nuclease analyses, the transcription start sites for early and late genes were mapped to positions 5115-5125 and 200-203, respectively (Daniel and Frisque, 1993). Overall, these studies suggested usage of different TATA boxes and start sites for early and late transcription of JCV.

There are two other examples of the genes containing dual TATA boxes. The CYC1 gene of the yeast Saccharomyces cerevisiae contains two functional TATA elements at sites -178 and -123 upstream of start site. The sequence near the 5′ end is ATATATATAT, whereas the sequence near 3′ the end is TATATAAAA. Further, it was suggested that the two TATA boxes recognized different factors of the transcription apparatus (Li and Sherman, 1991). The gene of Autographa californica

nuclear polyhedrosis virus (AcMNPV) is controlled by tandem promoters. Late transcripts initiate from conserved TAAG motif utilizing proximal TATA box, while early transcripts initiate from conserved CAGT motif using distal TATA element. Late transcripts were not affected by alterations in the distal TATA box. Similarly, early transcripts were not affected by mutations in the proximal TATA box. The results suggested the usage of different TATA boxes for early and late transcription, respectively (Guarino and Smith, 1992).

1.7.5.2 GLIAL CELL-SPECIFIC EXPRESSION OF JC VIRUS REGULATORY REGION IN CELL CULTURE

The first report identifying the promoter-enhancer signals of JCV was from Kenney et al (1984). They fused the regulatory sequences of JCV to the reporter gene, chloramphenicol acetyl transferase (CAT), in both orientations. The activity of the reporter plasmids was assayed in PHFG, HeLa, HEK, and CV-1 cells by performing CAT assay. Maximum activity was detected in PHFG cells. The construct in which the JCV promoter-enhancer sequences were cloned in the reverse orientation was about twice as active as the one cloned in the sense orientation. The activity observed in CV-1 cells was negligible. In contrast, the CAT construct with SV40 regulatory sequences was active in all the four cell types tested. Deletion of a single copy of the 98 bp repeat reduced the activity by 5-fold. Removal of all but the first

46 bp of the first tandem repeat totally abolished the activity. Deletion of one copy of the tandem repeat was more detrimental to the promoter-enhancer activity in PHFG cells. These results suggested that, unlike the other human polyoma viruses such as BKV, JCV has restricted glial cell-specificity.

In another study the regulatory region of JCV was replaced with that of either SV40 or BKV. The new hybrid constructs were not viable in human fetal glial cells. In the reverse hybrid constructs, the regulatory region of JCV controlled synthesis of either SV40 or BKV T antigen in human glial cells. These results suggested that SV40 and BKV T antigens could interact with JCV regulatory region but JCV T antigen, although shown to bind SV40 DNA, could not substitute for the homologous T protein. It also indicated that JCV T protein played a significant role in determining efficient gene expression in a cell-type-specific manner (Chuke et al, 1986).

Regulatory regions of type I and type II JCV variants isolated from PML patients were tested for their activity in PHFG and HEK cells. The activity of Mad1 strain was 15-fold more in PHFG cells than in HEK cells. The activity of Mad8Br-cat and the two Mad11-cat constructs showed only minor differences in their activity in PHFG cells. Mad7-cat and Her1-cat constructs failed to show detectable activity in PHFG

cells. The results with Her1-Br construct were surprising since the sequences are closely related to those of Mad8-Br. The differences in the activity of different JCV strains in PHFG cells were suspected to be due to differences in the number of promoter-enhancer elements in these strains. The suggestion that the presence of a second TATA box in the prototype MAD1 JCV regulatory region may interfere with the selection of the correct early mRNA start site was seriously considered in this case (Mandl and Frisque, 1986). However, no detectable difference was noticed in the activity of Mad1 and Mad4 strains (the latter not having the second TATA box). Hence the possibility of interference of selection of start site due to presence of a second TATA box was ruled out.

Further studies used additional cell types derived from primate and rodent species to address the mechanism of cell-specificity of JCV . A high level of expression was observed in glial cells but not in other cells. Quantitative S1 nuclease analysis showed higher levels of CAT transcripts, suggesting the high transcriptional activity in glial cells. In nonglial cell lines such as human cervical HeLa and monkey kidney COS cells the CAT activity was negligible and it was only 2-4 times more than the CAT construct lacking promoter-enhancer sequences (Feigenbaum et al, 1987). Taken together, the results strongly suggested that the expression of JCV is limited to glial cells.

A chimeric polyoma virus was constructed by inserting SV40 21 bp triplicates and 72 bp repeats at the distal end of the JCV regulatory region on the late side of 98 bp repeats without interrupting the coding potential of JCV T-antigen and capsid proteins. Although the chimeric virus replicated efficiently in human fetal glial cells, cell-induced deletions were found in the regulatory region. DNA sequence analysis revealed a 294 bp deletion from the original construct that retained one of the complete 98 bp repeats of JCV and one and one-third of the SV40 72 bp repeats. The other JCV 98 bp repeat and SV40 21 bp triplicates were consistently deleted. The virus produced JCV T-antigen without any changes in the amino acid sequence. The chimeric virus demonstrated wider species and cell-type host-range, producing infectious virus in human fetal brain and embryonic kidney as well as in rhesus monkey fetal and adult glial cells. However, the species host range was not extended beyond primate cells. These data suggested that the JCV regulatory sequences restrict the host range and the SV40 sequences were needed for the extended cell-type range. These sequences are responsible for the host range phenotype and the glial cell tropism (Vacante et al, 1989).

To further confirm the glial cell-specific expression of JCV, the promoter-enhancer sequences of the kidney-adapted variant of JCV, JCV-HEK-A were tested for activity. The

activity of CAT constructs containing the regulatory sequences of Mad1 and JC-HEK-A was tested in human kidney HEK and human neuroblastoma cell line, GOTO. Mad1 expressed only in GOTO cells, whereas, JC-HEK-A construct expressed in both the cell types. The results suggested that the regulatory region of JC-HEK-A might have undergone rearrangements and that the new DNA sequence inserts were able to interact with the factors present in HEK cells (SinoHara et al, 1989).

Two-phase partition assay for chloramphenicol acetyl transferase (CAT) was done to compare the activities of the JCV Mad1, BKV, and SV40 enhancer sequences in African green monkey kidney (CV-1) and owl monkey kidney (OMK) cells . The activity of Mad1 was 2.0 and 1.2% and BKV was 45 and 8% in CV-1 and OMK cells, respectively. This confirmed the restricted cell specificity of JCV (Martin, 1990). Recent studies compared the activities of Mad1, Mad8-Br, and Mad11-Br promoter-enhancer signals in human cervical HeLa cells, primary hamster fetal glial cell line POJ, and monkey kidney cell line SVG. In these cells, pMad1 exhibited only low activity, confirming the earlier observations of glial cell restricted expression of JC virus. Mad8-Br construct was active in all the three cell types. Mad11-Br construct was inactive in Hela cells, but 3.5- and 10-fold more active in POJ and SVG cells, respectively. The results suggested that the heterogeneity of the regulatory sequences of JCV may alter

the cell specificity of JCV promoter-enhancer under some conditions (Martin and Li, 1991).

1.7.5.2.1 EXPRESSION OF JCV IN P19 EMBRYONAL CARCINOMA CELLS

Previously, our laboratory used P19 embryonal carcinoma cells as a model system to study the glial cell-specific expression of JC virus. Embryonal carcinoma (EC) cells have been widely used as a model system for studying early murine development. EC cells are the malignant stem cells of teratocarcinoma and resemble the pluripotent cells from the inner cell mass of early mouse preimplantation embryos (Martin, 1980). A number of differentiated cell types, with characteristics of cells of the three germ layers can be obtained by different treatments (Martin, 1980). When implanted into mouse embryos, EC cells can contribute to the development of normal embryos. This suggests that the mechanisms of differentiation of EC cells are similar to those of normal embryonic cells (McBurney et al, 1988).

Addition of retinoic acid (RA) at 10^{-6} M to monolayer cultures of P19 EC cell line induces differentiation to a heterogeneous population consisting of endoderm- and mesoderm-like cells. These cells are fibroblast-like and few neurons are also found (Jones-Villeneuve et al, 1983). When induced to differentiate with 300nM RA, P19 EC cells develop into cells resembling those of embryonic brain tissue. Addition of RA at 300 nM to aggregating P19 cells induces heterogeneous

population of neuronal cells consisting of neurons, astroglia, microglia and fibroblast-like cells (McBurney, 1993). Neurons and glial cells in RA-treated P19 cells are always found together with the neurons usually lying on top of the glial cell monolayer. The fibroblast-like cells are seldom covered with neurons (Jones-Villeneuve et al, 1983; Rudnicki and McBurney, 1987). There is no evidence that the neuronal cells induced by RA are mature brain glial cells. However, these cells are a mixture of glioblasts, astrocytes and microglia in various stages of differentiation. P19 EC cells can be differentiated into skeletal and cardiac muscle cells when treated with 1% dimethyl sulfoxide (DMSO).

Previous studies from our laboratory suggested that the expression of regulatory region of JCV was seen only in RA-treated but not in DMSO-treated or undifferentiated P19 cells. (Nakshatri et al, 1990a). The P19 cells are a good system to demonstrate the mode of tissue-specific regulation of JCV expression. These cells can be differentiated into different cell types from an identical genotype. Hence, I used P19 cells in my studies. Earlier studies suggested that JCV is expressed efficiently in glial cells (Major et al, 1992). The neural cells induced after treating P19 cells consist of a heterogeneous population including glial cells. This is the disadvantage of the P19 cell system. However, previous studies from our laboratory reported efficient expression of JCV

regulatory region in RA-treated P19 cells. I also used nuclear and whole cell extracts prepared from RA-treated P19 cells in several *in vitro* studies presented in this thesis. Since RA-treated P19 cells contain a heterogeneous population of neuronal cells, the exact cell type in which JCV is expressed is uncertain. However, it is evident from the existing literature that the expression of JCV is restricted to glial cells.

1.7.5.3 CELLULAR TRANSCRIPTION FACTORS THAT BIND TO JCV PROMOTER-ENHANCER SEQUENCES

The studies mentioned above clearly indicated that the expression of JCV is restricted to glial cells. Further, cell culture studies, using different strains of JCV, indicated that the glial cell specific expression of JCV is not due to the cell-specific adsorption or penetration of the virus. Cell-specific expression was due to the intracellular factors that bind to the sequences in the regulatory region of JCV (Frisque *et al*, 1979). Hence subsequent studies including those in this thesis were focused on finding the sequences that are bound by the transcription factors in the glial cells.

Using the oligonucleotides that span the 98 bp tandem repeat of JCV and the nuclear extracts from human fetal brain and HeLa cells, mobility shift and UV crosslinking assays were performed to identify the sequences that confer tissue tropism

and the cellular factors that bind these sequences (Khalili et al, 1988). Complementary oligonucleotides having homology to NF1 binding site that span nt 134-160 of the 98 bp repeat of JCV interacted with proteins of 45 and 85 kDa from human fetal brain and HeLa cells, respectively. Oligonucleotide sequences complementary to TATA box interacted with a protein of 78/80 kDa. The oligonucleotide having sequences homologous to the 5' and 3' ends of promoter-enhancer bound similar proteins of 82 and 78/80 kDa from both extracts. However, a protein of 230 kDa from both extracts interacted with several regulatory sequences of promoter-enhancer.

DNaseI footprinting experiments on JCV promoter-enhancer were conducted using mouse brain nuclear extracts. The protected sequences in the 98 bp repeats corresponding to nt 35-58 and 133-156 were homologous to nuclear factor 1 (NF1) binding sites. The second set of sequences that were protected corresponding to nt 208-229 have a pseudo-NF1 motif (Tamura et al, 1988).

Mobility shift assays with JCV regulatory region showed binding activity with the nuclear extracts from HeLa, PHFG, and human glioma cell line, A172. However, no binding was detected with extracts from HEK cells. Further, DNaseI footprinting was carried out on JCV regulatory region using extracts from two glial-type cells and Hela cells. Similar sequences and NF1 binding motifs were protected as observed

earlier by Tamura et al (1988). Competition experiments with the NF1 binding site oligonucleotide of adenovirus replication origin confirmed that the protected sequences were NF1 motifs (Amemiya et al, 1989).

DNaseI footprinting assay using extracts prepared from undifferentiated (UD), DMSO-differentiated and RA-differentiated P19 EC cells was attempted to detect the proteins interacting with the sequences in JCV enhancer (Nakshatri et al, 1990a). Protection of three regions, two in the 98 bp repeats and one towards the late side of the repeat was observed with extract from RA-differentiated but not with extracts from UD and DMSO-differentiated P19 cells. All these sites contained sequences resembling NF1 binding motifs. The sequences protected within the 98 bp repeats were named regions II and III, respectively, whereas the region of sequences protected outside the repeats was called region I. In addition to NF1 binding site, region I also contained a binding motif for E4TF1 factor (Fig. 1.2). Competition experiments with oligonucleotides corresponding to regions I and II suggested that the same factor binds to all the three NF1 binding motifs, although they consist of different sequences. A suggestion that the NF1 I binding site located outside the repeats may play a role in the cell-specific expression of JCV was offered based on competition experiments (Nakshatri et al, 1990a).

Another factor which binds to a sequence overlapping the NF1 motifs located in the repeats called SacI motif was also reported. It was suggested that the cooperativity of NF1 motif and SacI motif binding factors may be required for the strong brain specificity of JCV (Tamura et al, 1990b).

The 45 kDa protein from human fetal glial cells that interacts with the sequences corresponding to nt 134-160 NF1 motif of JCV enhancer was purified and it complemented for the activity of JCV enhancer in HeLa cells in in vitro transcription assays (Ahmed et al, 1990). Subsequently the cDNA encoding the factor binding to NF1 motif in the 98 bp repeat was cloned. Expression of this cDNA increased the transcription of the JCV late promoter-enhancer and to a lesser degree the early promoter-enhancer (Kerr and Khalili, 1991).

A glial cell transcription factor belonging to the POU-domain family, Tst-1 stimulated transcription of both early and late viral genes. Tst-1 also stimulated viral DNA replication and hence it was suggested that Tst-1 is one of the determining factors in the glial cell-specificity of JCV (Wegner et al, 1993). Presence and role of a binding site homologous to cyclic AMP response element (CRE) adjacent to NF1 II/III in JCV promoter-enhancer in the regulation of JCV was speculated in another study (Amemiya et al, 1992). A pentanucleotide repeat, AGGGAAGGGA, present between the NF1

II/III and TATA box interacts with an approximately 56 kDa protein from glial and nonglial cells and down regulates the JCV late promoter activity (Tada et al, 1991).

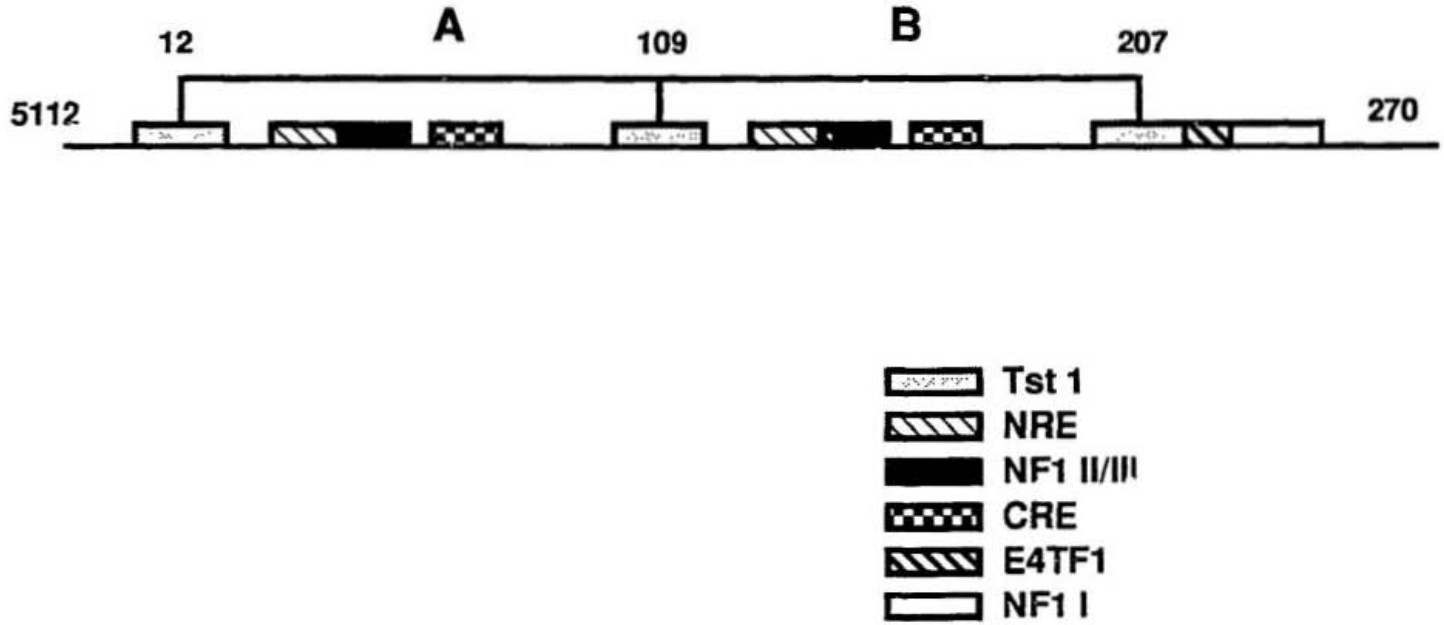
Overall, it is evident that JCV promoter-enhancer is regulated by both positive and negative regulatory mechanisms. The binding sites for the various transcription factors in the JCV control region are diagrammatically represented in Fig. 1.2.

All the studies mentioned above used in vitro binding assays to suggest that NF1 may play a role in glial cell-specific expression of JCV. Moreover, in the study reported by Wegner et al (1993), cotransfection of a recombinant plasmid expressing a brain-specific factor Tst 1 with the reporter plasmid containing JC early regulatory region activated JCV_E expression in glial cells. In my opinion, this study should have shown the role of Tst 1 in glial cells as such. In addition, studies on late gene expression of JCV, which is important for the productive infection of the virus are lacking. The regulatory region of JCV contains several cis-acting elements (Fig. 1.2). At present there is no study that explains how the proteins binding to these elements function in glial cell-restricted expression of JCV.

1.8 THESIS RATIONALE AND ORGANIZATION

The purpose of my study is to understand the role of factor(s) binding to the NF1 motif and cyclic AMP response

Fig. 1.2. Diagrammatic representation of binding sites for various transcription factors in the regulatory region of JCV. The letters A and B and large boxes indicate the 98 bp repeats. The numbers denote the nucleotide positions. Binding sites for cellular transcription factors are indicated. The early JCV_E and late JCV_L promoters are on the left and right, respectively.


12
A
109
B
207
5112
270
Tst 1
NRE
NF1 II/III
CRE
E4TF1
NF1 I

element (CRE) located adjacent to the NF1 sequence in the 98 bp repeats in in vivo activity of early gene expression of JCV and to correlate the in vivo activity with in vitro DNA-protein interactions. It is also the objective of my study to address the mechanism of regulation of JCV late gene expression. In addition, I have attempted to determine how the proteins binding to cis-acting elements located in the 98 bp repeats function in regulating JCV gene expression.

The thesis is organized in five chapters. In chapter 2, I will describe materials and methods used for the work presented in chapters 3, 4 and 5. In chapter 3, I will detail the role of NF1 binding motifs in glial cell-specific expression of JCV and correlate the in vivo activity with in vitro DNA-protein interactions. Additionally, the isolation and preliminary characterization of a cDNA encoding a factor binding to nuclear factor 1 (NF1) located within the 98 bp repeats will be described.

The early gene product of JCV, T-antigen transactivates the JCV late promoter leading to productive infection of JCV. In chapter 4, I will detail the role of NF1 binding sites in the transactivation of JCV_L and the delineation of amino acid sequences of T antigen required for transformation and transactivation of JCV_L. A model for transactivation of JCV_L by T antigen will be presented.

In chapter 5, I will detail the role of cyclic AMP response element (CRE) in glial cell-specific expression of JCV. The characterization of protein(s) binding to this sequence is also presented.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

The restriction endonucleases were purchased from New England Biolabs and Bethesda Research Laboratories (Gibco-BRL). T4 DNA ligase, calf intestinal phosphatase (CIP) and reverse transcriptase were from BRL, BRL and Life Sciences, respectively. The [^{14}C] chloramphenicol and [^{32}P]dCTP were obtained from ICN and Amersham, respectively. Acetyl coenzyme A was from Sigma. Thin layer chromatography (TLC) plates and X-ray films were purchased from Kodak.

Synthetic oligonucleotides were purchased from Regional DNA Synthesis Laboratories, University of Calgary. The XbaI translation termination linker used in generating JCV large T-antigen mutants described in Fig. 5.7 was commercially obtained from New England Biolabs. The mutagenesis kit was supplied by Bio-Rad. The plasmid pSV0cat was a kind gift from Dr. B. Howard. The JC early promoter-enhancer reporter plasmid pJC$_F$cat was constructed as described (Nakshatri et al, 1990a). The plasmid pBLcat2 was a generous gift of Dr B. Luckow.

The (Nick-column) Sephadex G-50 columns were obtained from Pharmacia. Protease inhibitors like antipain, pepstatin, aprotinin, and PMSF were purchased from Sigma. DNaseI, alpha-amanitin, forskolin, dibutyryl cyclic AMP, and 5' bromo deoxyuridine (BUdr) were from Sigma. The chemicals 5-bromo-4-

chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT), and alkaline phosphatase-tagged anti-mouse IgG secondary antibodies used in western blotting were purchased from Sigma chemicals and Bio-Rad, respectively. Poly dI.dC was purchased from Pharmacia. The nick-translation kit was supplied by Gibco-BRL. Circle nitrocellulose filters were obtained from Mandel Scientific Company. The mRNA isolation kit was from Pharmacia. The cDNA synthesis, and λ packaging kits were purchased from Gibco-BRL.

Alpha-MEM medium and phosphate buffered saline (PBS) were supplied by Flow Laboratories. Trypsin-EDTA, all-trans retinoic acid were from Sigma. Dimethyl sulfoxide (DMSO) was purchased from Baker. The mouse embryonal carcinoma P19 cell line was a kind gift from Dr. Hamada. Bockneck Laboratories supplied fetal calf serum (FCS). The monoclonal antibodies against SV40 T antigen, PAb 108 and MHC class II antigen, I-A^k were obtained from American Type Culture Collection (ATCC).

2.2 METHODS

2.2.1 Cell culture, differentiation of embryonal carcinoma cells, and transfection

The embryonal carcinoma cell line, P19, was cultured in alpha-MEM medium with 10% heat-inactivated fetal calf serum and cells were induced to differentiate as described (Rudnicki and McBurney, 1987). Briefly, cells were trypsinised, resuspended in media containing either 300 nM all-trans

retinoic acid (RA) or 1% dimethyl sulfoxide (DMSO) and then cultured in bacterial culture plates for 48 h. Fresh media containing RA or DMSO was added after 48 h and cells were incubated for another 48 h. After four days of treatment the cell aggregates were washed in phosphate buffered saline, trypsinised and resuspended in media containing no RA or DMSO and plated in tissue culture plates. Eight hours after plating, the cells were transfected with 10 μg of indicated test plasmids and 10 μg of pUC19 DNA by calcium phosphate precipitation (Graham and van der Eb, 1973). For the cAMP induction experiments described in chapter 5, the cells were transfected with 5 μg of indicated plasmids and 15 μg of pUC19 DNA unless and otherwise mentioned. Twenty four hours after transfection, the cells were incubated with medium in the absence or presence of 100 μM forskolin and 10 μM dibutyryl cAMP.

For the studies on transactivation of JCV late promoter by T-antigen, the cells were transfected with 1 μg of indicated reporter plasmid with either 10 μg of CMV-JCV-TA T-antigen expression plasmid or pUC19 DNA. Primary 5-day-old Fischer baby rat kidney (BRK) cells were used in the transformation assays with JCV T-antigen as described (Pater et al, 1988). Briefly, the cells were plated in the 60 mm tissue culture dishes and the indicated wild type and mutant T-antigen plasmids were co-transfected with EJ-ras oncogene.

After two days the medium containing 2% fetal calf serum was added and the cells were subsequently maintained in this medium for three weeks. Then the transformed foci were stained with crystal violet and counted.

2.2.2 Plasmids and site-directed mutagenesis

Wild type pJC_Lcat is the same as pJC_E cat except that the regulatory sequences of JCV are in reverse orientation (nt 5112 to 270) and as described earlier (Nakshatri et al, 1990a). Site-directed mutagenesis of JCV enhancer was performed with a kit from Gibco-BRL, according to the manufacturer's instructions. The JCV enhancer Pvu II to Hind III fragment from 270 to 5112 served as the substrate for mutagenesis. Mutations in the Nuclear Factor 1 (NF1) binding sites were generated using complementary strand-homologous oligonucleotides for nt 235-206 for region I, 165-137 for the two nt substitution mutations for region II and 152-129 for the remaining region II and III mutations, with appropriate base substitutions. To introduce mutations into cyclic AMP response element (CRE), a complementary strand-homologous oligonucleotide for nt 142-169 with appropriate base substitutions was used. The mutations were confirmed by dideoxy sequencing (Sanger et al, 1977).

The mutated enhancer fragments (Hind II-Sma I) were inserted into the Hind III site of pSV0CAT such that the chloramphenicol acetyltransferase (CAT) gene is expressed from

the early JCV promoter (Nakshatri et al, 1990a). The constructs were designated for the NF1 and CRE site(s) that were mutated. The sequences mutated and the names of the plasmids are shown in Figs 3.1 and 4.1 for NF1 mutations and in Fig. 5.5 for CRE mutations.

The plasmids pRII and pmRII contain the single 98 bp repeat having wild type and mutated NF1 II sequences, cloned into Hind III site of pSV0 cat. For pJCV-CREcat, the AGCATGAGCTCATACC, JCV CRE oligonucleotide was cloned into Sal I site of pBLcat2 expression vector. Insertion was confirmed by DNA sequencing.

The CMV-JCV-TA plasmid for expressing JCV T-antigen consists of JCV large T-antigen coding sequences (nt 5013 to 2603) under the control of cytomegalovirus (CMV) promoter-enhancer sequences as described previously (Nakshatri et al, 1990a). The translation termination linker and deletion mutations were derived from CMV-JCV-TA. Plasmids were numbered for the sites of termination and the sizes of deletions. Plasmids pK4984, pK4246, pK4129, pK4085, pK3359, pK3013, and pK2896 were generated with termination linkers having a termination codon in all of the three reading frames, at Nco I, BglII, SspI, SspI, PstI, StuI, and PvuII sites, respectively. Deletion mutations of the BglII-SspI (pK 117), SspI-PstI (pK 726), and StuI (pK 1945) fragments were generated (Fig. 4.7).

The plasmid, pCMVNF1 contains the cDNA isolated from the cDNA library prepared from P19 RA cells after screening with NF1 binding site located in the 98 bp repeats of JCV. The cDNA from the recombinant λK1 phage was liberated by digesting with NotI and SalI restriction enzymes and cloned in NotI and HindIII sites of pRC/CMV vector purchased from Invitrogen. The insertion and orientation was confirmed by sequencing. The plasmid pBK_E cat contains the regulatory region of human BK virus in early orientation as described previously (Pater and Pater, 1988).

2.2.3 Chloramphenicol acetyltransferase (CAT) assays.

Cells were harvested for CAT assays 48 hours after transfection as described (Gorman et al, 1982). Briefly, the cells were washed with PBS, scraped into eppendorf tubes with a rubber policeman, and pelleted. The pellet was resuspended in 0.25 M Tris-Hcl (pH 7.8) and subjected to three cycles of liquid nitrogen freezing and thawing at 37^0 C. The cell debris was pelleted and the supernatant was used in CAT assays.

Ten μl of the extract was incubated at 37^0 C for 1 hour in a reaction mixture containing 14 μl of 1 M Tris-Hcl (pH 7.8), 4 μl of 4 mM Acetyl coA, 1 μl of distilled water, and 1 μl of [^{14}C] chloramphenicol. After 1 hour of incubation, the chloramphenicol was extracted by adding ethyl acetate and then dried in a speed vacuum. The samples were resuspended in 15 μl of ethyl acetate and spotted on TLC plates. Acetylated and

non-acetylated forms of chloramphenicol were separated by ascending TLC in chloroform:methanol (95:5). Percentage acetylation was determined by liquid scintillation counting. The β-galactosidase control plasmid was from pCH110 (Pharmacia) in which we replaced SV40 with Rous sarcoma virus (RSV) promoter-enhancer sequences. This served as a control for transfection efficiency. % conversion on CAT assay figures indicate the % CAT activity.

2.2.4 Nuclear extract preparation and DNase I footprinting

The preparation of nuclear extract and DNase I footprinting were done as described previously (Hennighausen and Lubon, 1987). Briefly, the cells from 40 plates of undifferentiated or RA-differentiated cells were used for nuclear extract preparation. The cells were washed with PBS and then scraped with a rubber policeman and pelleted by centrifugation at 1800 rpm at 4^0 C for 10 minutes. The pelleted cells were again washed in 10 volumes of PBS and then resuspended in 5 volumes of 0.3 M sucrose in buffer A (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM of protease inhibitor phenylmethylsulfonyl fluoride (PMSF), and 2 μg/ml each of other protease inhibitors antipain, pepstatin A, and aprotinin). Cells were homogenised in a Dounce glass homogenizer with a B pestle by applying 10-12 strokes without and 1-2 strokes with 0.3-0.4% of Nonidet P-40 (NP-40). The nuclei were then pelleted by centrifugation at 2700 rpm at 4^0

C and washed twice with buffer A containing 0.3 M sucrose but no NP-40.

The nuclei were resuspended with a dounce homogenizer by applying 10 strokes in 2.5 pelleted nuclei volumes of buffer B (400 mM NaCl, 10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 5% glycerol and 0.5 mM PMSF). The resuspended nuclei were gently stirred at 4^0 C for 30 minutes and then centrifuged for 1 h at 39,000 rpm at 4^0 C. The supernatant was dialysed for 4 h at 4^0 C against 50 volumes of buffer C (20 mM HEPES-KOH pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol and 0.5 mM PMSF). After 4 hours of dialysis, the material which was precipitated during dialysis was removed by centrifugation at 20,000 rpm at 4^0 C. The supernatant was aliquoted, flash frozen in liquid nitrogen, and stored at -70^0 C until further use. The protein concentration was estimated by the method of Bradford (1976).

To generate probe for DNaseI protection assays, the Hind III to PvuII fragment of JCV (nt 5112 to 270) was cloned in XbaI site of pUC19 vector. The SalI to SmaI fragment containing the insertion was used in the footprinting assay. The SalI to SmaI fragments of mutated enhancers eluted from the vector pTZ19 used in *in vitro* mutagenesis were also included in the footprinting assay. The DNA fragments were end-labelled by using reverse transcriptase in the presence of alpha [^{32}P] dCTP which fills the SalI ends. The end-labelled

DNA fragments were separated from the non-incorporated nucleotides through Sephadex G-50 columns.

Nuclear extracts in 15-20 µl volumes (containing 45-60 µg of protein) were incubated in 50 µl of binding buffer (50 mM NaCl, 0.1 mM EDTA, 20 mM HEPES-KOH pH 7.5, 0.5 mM DTT, 10% glycerol) and one µg of poly (dI.dC) for 15 minutes on ice. The end labelled probe (40,000 cpm) was added and incubated at 20^0C for 10 minutes. $MgCl_2$ and $CaCl_2$ were added to a final concentration of 5 and 1 mM, respectively. Pancreatic deoxyribonuclease (DNase) I at a concentration of 2 µg/µl in a solution containing 150 mM NaCl and 50% glycerol was diluted with 25 mM NaCl, 10 mM Hepes-KOH pH 7.5 and 0.5 mM DTT. The reaction containing no protein (in lanes marked as free in the figures) contained 2.0 and 1.0 ng of DNase I. The probes incubated with the proteins contained 240 ng of DNase I. After 30 seconds of digestion with DNase I, the reactions were stopped by adding 100 µl of stop buffer (0.375% SDS, 15 mM EDTA, 100 mM NaCl, 100 mM Tris-HCl pH 7.6, 50 µg/ml of sonicated salmon sperm DNA, and 100 µg/ml pronase). The samples were incubated at 37^0 C for 15 minutes and 2 minutes at 90^0 C followed by phenol-chloroform-isoamylalcohol extraction and ethanol precipitation. The reaction products were analyzed on polyacrylamide-8M urea sequencing gels.

2.2.5 Whole cell extract preparation

Whole cell extract was prepared by the method described

previously (Tasset et al, 1990). Briefly, the cells were washed with PBS and scraped with a rubber policeman and pelleted by centrifugation at 1800 rpm at 4^0C for 10 minutes. The pelleted cells were washed again with 10 volumes of PBS and suspended in 2 pelleted volumes of buffer containing 10 mM Tris pH 7.6, 1 mM EDTA pH 8.0, 400 mM Kcl, 1.5 mM $MgCl_2$, 20% glycerol, 1 mM DTT, 10 μg/ml of soya bean trypsin inhibitor (SBTi), 1 mM PMSF, and 2 μg/ml of each of protease inhibitors antipain, pepstatin A, leupeptin, and aprotinin. This was followed by three cycles of freezing in liquid nitrogen and thawing on ice and centrifugation for 1 h at 39,000 rpm at 4^0C. The supernatant was aliquoted, flash frozen in liquid nitrogen, and stored at -70^0C for further use. The protein concentration was estimated by the method of Bradford (1976). The whole cell extracts expressing JCV T-antigen were prepared from P19 RA cells transfected with 20 μg of CMV-JCV-TA plasmid.

2.2.6 Sequences of oligonucleotides used in in vitro binding assays.

NF1 I	5′-AAGGGGAAGTGGAAAGCAGCCAA-3′ 3′-TTCCCCTTCACCTTTCGTCGGTT-5′
NF1 II/III	5′-GTCGACTGGCTGCCAGCCAA-3′ 3′- ACCGACGGTCGGTTGTCGAC-5′
Mutant NF1 II/III	5′-GGAGTACTGCCAGACCAG 3′- CATGACGGTCTGGTCCCT-5′

JCV CRE 5'-AGCATGAGCTCA-3'
3'- ACTCGAGTTCGT-5'

2.2.7 Mobility shift assays.

The end labelling of enhancer fragment of JCV and oligonucleotides used in mobility shift assays was the same as that used in DNase I footprinting mentioned in 2.2.4. Since annealing of the complementary strand oligonucleotides generates an overhang, the end labelling was done using reverse transcriptase as described in 2.2.4.

The nuclear and whole cell extracts were used in mobility shift assays as indicated in figure legends. Mobility shift assays were done as described previously (Chodosh et al, 1988). Briefly, the binding reactions in 15 µl volume were done in buffer containing 12 mM HEPES-NaOH pH 7.9, 4 mM Tris-HCl pH 7.9, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 5 µg of poly (dI.dc).poly (dI.dC), 60 mM KCl, 15,000 cpm of end-labelled probes and the indicated amounts of nuclear or whole cell extracts. The reactions were incubated at 20^0 C for 30 minutes. The lanes labelled as free in the figures did not contain protein. The reaction products were analyzed on 4% non-denaturing polyacrylamide (acrylamide:bisacrylamide, 29:0.5) gels which were run at 4^0 C with circulation of buffer at 100v in 22 mM Tris-borate, 0.5 mM EDTA. Gels were dried and subjected to autoradiography.

2.2.8 UV crosslinking assays

UV crosslinking assays were done as described (Chodosh et al, 1986). Binding reactions in 35 µl volume were done as described for mobility shift assays except that 100,000 cpm of BudR incorporated, nick translated JCV CRE oligonucleotide, 10 µg of poly dI.dC, and 60 µg of whole cell extract were used. After incubation for 30 min at 30°C, the reaction mixtures were exposed to UV irradiation under the Fotodyne UV lamp for 30 min. The reactions were not treated with DNase I as the size of the probe was less than 50 bp. The reactions were stopped by adding 2X SDS-PAGE loading buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol, and 0.2% bromophenol blue). The products were boiled for 4 minutes and then subjected to electrophoresis on a 10% SDS-polyacrylamide gel at a constant voltage of 100 volts.

2.2.9 Southwestern blot analysis

Southwestern blotting was done according to the method described previously (Vinson et al, 1988). Fifty µg of protein from forskolin (FSK)-treated and untreated whole cell extracts prepared from RA-differentiated P19 cells was run by electrophoresis on a 10% denaturing SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane by electrotransfer in Tris-glycine buffer. The filters were blocked in binding buffer containing 5% non-fat dry milk in 25 mM Hepes-NaOH pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT, 25 mM NaCl at room temperature

(RT) for 2 h. This was followed by incubation in the binding buffer containing 10^6 cpm per ml of nick-translated JCV CRE probe at RT for 2 h. The membranes were then washed thrice in binding buffer, 15 minutes each. Subsequently, the membranes were air dried and exposed to X-ray film for autoradiography.

2.2.10 RNA preparation and ribonuclease (RNase) protection assays

RA-treated P19 cells transfected with and without 10 μg of pJC_E cat plasmid were harvested for RNA 48 h after transfection (Chirgwin et al, 1979). Total RNA at 10 μg was used in RNase protection experiments as described (Belaguli et al, 1992). The RNA was hybridized with 100,000 cpm of cat and β-galactosidase RNA probes at 56^0C for 14 h followed by RNase T1 (700 U of RNase T1 per ml) digestion. RNase resistant hybrid RNAs were analyzed on 5% polyacrylamide-8M urea sequencing gels. The quantitation of the signals was done by laser densitometry. The cat and β-galactosidase probes were generated from the HindIII-EcoRI DNA fragment (nt 5018-4768) of pSV2 cat and EcoRI-AvaI fragment (nt 3286-3063) of pRSV β-galactosidase DNA cloned in the vector, Bluescript KS+, respectively.

2.2.11 *In vitro* transcription assays

Nuclear and whole cell extracts were prepared from UD and RA-differentiated P19 cells as described in 2.2.4 and

2.2.5, respectively. In vitro transcription was done as described earlier (Ahmed et al, 1990). Transcription reactions were prepared in 50 µl volume containing 12 mM Hepes-NaOH pH 7.9, 12% glycerol, 60 mM KCl, 2 mM $MgCl_2$, 1 mM each of ATP, CTP, GTP and 0.5 mM of ^{32}P UTP, 1 µg of DNA template digested with appropriate restriction enzyme and the indicated amounts of protein. After incubation at 30^0 C for 1h, the samples were extracted with phenol-chloroform-isoamylalcohol and ethanol precipitated. The run-off transcripts were analyzed on a denaturing 5% polyacrylamide-8M urea gel with the appropriate size markers. The DNA templates were 1 µg of Nco I and Pvu II digested JC early and late cat constructs to yield 550 and 160 nt run off transcripts, respectively. In cAMP induction experiments described in chapter 5, 1 µg each of Nco I digested pRII and pmRIIcat DNA templates were used to obtain 550 nt run off transcripts. pBLcat2 and pJC-CREcat DNA were digested with Nco I to yield 576 nt transcripts. The Hpa I digested pRSV-ß-galactosidase plasmid was used as an internal control to normalize the recovery of transcripts.

2.2.12 Preparation of cDNA library from RA-differentiated P19 embryonal carcinoma cells

2.2.12.1 Isolation of poly A+ RNA

Poly A+ RNA from P19 RA cells was prepared using the mRNA isolation kit of Pharmacia according to the manufacturer's instructions. Briefly, the cells were washed

with sterile PBS and pelleted down by centrifuging at 1800 rpm at 4^0 C. Lysis buffer (100 ml) was added to the pellet. The pellet was resuspended and sheared by forcing the lysate in and out of an 18 gauge needle until viscosity reached that of the lysis buffer.

The lysate was then incubated for 2 hours at 45^0C with intermittent agitation. The NaCl concentration of the lysate (0.2M) was adjusted to 0.5M and then added to oligo(dT) cellulose equilibrated with binding buffer and incubated for 30-60 minutes at room temperature with intermittent agitation. The resin was pelleted and washed with 5 bed volumes of binding buffer until the buffer was clear. The washed resin was poured into column and washed continuously until the A_{260} of eluate was less than 0.05. Then, the bound poly A+ mRNA was eluted with 0.3-0.5 ml fractions of elution buffer. The fractions 1-8 containing majority of the mRNA were pooled and precipitated by adding 0.1 volume of 3M NaOAc and 2 volumes of 100% ethanol at -20^0 C overnight. The mRNA was recovered by centrifugation at 4^0 C. The amount of mRNA recovered was quantitated by UV spectrophotometry and absorbence readings at wave lengths of 260 and 280 nM were taken to estimate the purity of the mRNA and confirmed the purity.

2.2.12.2 Preparation of cDNA from mRNA

The cDNA was prepared from the mRNA isolated from P19 RA-treated cells, using a kit from Gibco-BRL and according

to the manufacturer's instructions. Five μg of mRNA was added to 1 μg of NotI primer-adapter containing 15 dT residues. The mixture was heated to 70^0 C for 10 min, and quick-chilled on ice. Then, the 5X first strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT and 500 μM each of dATP, dCTP, dGTP, and dTTP) was added. This was followed by the addition of 2 μl (200 U/μl) of Superscript RT in a reaction volume of 20 μl and incubation at 37^0 C. After 1 hour of incubation, the mixture was placed on ice and the synthesis of second strand was initiated. To the first strand mixture, 30 μl of 5X second strand buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 5mM $MgCl_2$, 10 mM $(NH4)_2SO4$, 0.15 mM β-NAD^+ 250 mM each of dATP, dCTP, dGTP and dTTP, 1.2 mM DTT) was added. E.Coli DNA ligase (10 U), E.Coli DNA polymerase I (40 U) and E.Coli RNase H (2 U) were subsequently added in a total reaction volume of 150 μl and incubated at 16^0 C for 2 hours. After 2 hours of incubation, 10 U of T4 DNA polymerase was added and incubation was continued at 16^0 C for 5 min. The reaction was stopped by adding 10 μl of 0.5 M EDTA. The reaction products were subjected to phenol-chloroform-isoamylalcohol extraction followed by ethanol precipitation.

2.2.12.3 Size fractionation of cDNA and ligation of cDNA to λgt22A vector

The cDNA was checked on denaturing alkaline agarose gel before proceeding to the subsequent steps. SalI

linker was added at the 5′ end of cDNA. Then, the cDNA was digested with NotI and size-fractionated by column chromatography. This removed the residual adapters and the NotI fragments released by restriction digestion of primer-adapter with NotI. The size-fractionated cDNA containing SalI and NotI termini was ligated to λgt22A vector digested with NotI and SalI to ensure the preparation of a unidirectional cDNA library.

2.2.12.4 Packaging of phage λ vector-cDNA and preparation of cDNA library

The ligated vector-cDNA was then packaged using BRL's λ packaging system. Briefly, the 5 μl of the ligated mixture was added to the extract A supplied by BRL packaging kit. Then, 15 μl of extract B of the kit was added and mixed thoroughly but gently and incubated at 20^0 C for 2 h. Following the incubation, 0.5 ml phage dilution buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 0.01%(w/v) gelatin) was added and mixed. Chloroform (20 μl) was added next and mixed gently. The phage mixture was mixed with the Y1090R$^-$ host bacteria in phage dilution buffer and incubated at 37^0 C for 30 min. Three ml of molten 0.7% T-Tyn agar containing 10 mM $MgCl_2$ was then added, mixed and poured onto 100 X 15 mm 1.5% T-Tyn agar plates. After the top agar was set the plates were incubated at 37^0 C overnight. The phage library consisted of 1.1 X 10^6 plaques.

2.2.12.5 Amplification of cDNA library

The plates containing approximately 50,000 plaques each were overlaid with 10 ml of phage dilution buffer and incubated at 4^0 C overnight with gentle rocking. The bacteriophage suspension from each plate was recovered and pooled into sterile tubes. Chloroform was added to 5% final concentration and mixed and incubated for 15 min at room temperature. The cell debris was removed by centrifugation for 5 min at 4000 X g. The supernatant was transferred to fresh tubes and chloroform was added to 0.3% and the aliquots were stored in aliquots at 4^0 C for further use. After amplification, the titre of the library was 10^{11} pfu/ml.

2.2.13 Screening of cDNA library

The P19 RA λ gt 22A library was screened by Southwestern blot analysis to isolate cDNA encoding the factor interacting with NF1 II/III of JCV (Vinson et al, 1988). Briefly, the bacterial strain Y1090R$^-$ was infected with recombinant phages at 37^0 C for 30 min. Then, 3 ml of 0.7% top agar was plated over the 1.5% T-Tyn agar plates which were then incubated at 42^0 C for 4h. The IPTG-impregnated nitrocellulose membranes were overlaid on the plates and incubated for 6 hours at 37^0 C. The filters were then immersed in binding buffer (25 mM NaCl, 5 mM $MgCl_2$, 25 mM HEPES, pH 8.0, and 0.5 mM DTT) containing 6M guanidine hydrochloride and were gently shaken at 4^0 C for 15 min. The guanidine hydrochloride was

sequentially diluted with an equal volume of binding buffer and filters were immersed in this buffer for 5 min each. This process of denaturation and renaturation was repeated four times.

The filters were then immersed twice in binding buffer without guanidine hydrochloride twice for 15 min each time. Following this, the filters were submerged in binding buffer with 5% nonfat dry milk powder for 2 hours. The filters were further incubated in binding buffer containing 0.25% nonfat dry milk powder and 10^6 cpm/ml of nick translated ten-mer of NF1 II/III oligonucleotide for 2 hours. Finally, the filters were washed in binding buffer with 0.25% nonfat milk powder three times for 10 min. The filters were dried and subjected to autoradiography.

2.2.14 Western blot analysis

Western blotting was performed as described previously (Harlow and Lane, 1988). Briefly, the lysates prepared from P19 RA cells transfected with either wild type (WT) or mutant T-antigen expression plasmids were separated on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes electrophoretically. The membrane was immersed in Tris buffered saline (TBS; 100 mM Tris HCl pH 7.5, and 0.9% NaCl) containing 5% nonfat dry milk overnight. The membrane was washed five times for 20 minutes each wash with TBS containing 0.1% Tween 20 (TTBS). This was followed by

incubation in TTBS containing PAb108 monoclonal antibody against T-antigen for 2 hours. After 5 washes of 20 minutes each in TTBS, the membrane was incubated for 1 h with the alkaline phosphatase-linked anti-mouse IgG secondary antibody in TTBS. The membrane was washed five times with TTBS and then incubated with detection reagents, 0.5% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.5% nitroblue tetrazolium (NBT), for colour development until the bands were suitably dark. The reaction was stopped by washing with stop solution (20 mM EDTA in phosphate buffered saline) and the membrane was air dried.

2.2.15 Northern blot analysis

Northern blot analysis was done according to standard molecular biology procedures (Sambrook et al, 1989).

CHAPTER 3

Human JC virus perfect palindromic nuclear factor 1 (NF1) binding sequences important for glial cell-specific expression in P19 differentiating embryonal carcinoma cells.

3.1 INTRODUCTION

As discussed in the thesis introduction, cis-acting enhancer elements interact with cell-specific transcription factors to regulate expression of specific cellular genes. Unlike the other human polyoma virus BK, JC virus exhibits strict cell-specificity and its growth and expression is restricted to glial cells (Kenney et al, 1984; Small et al, 1986). The restricted cell-specificity was attributed to the transcriptional control region of JCV (Nakshatri et al, 1990a).

Nucleotide sequence analysis of brain and kidney forms of JCV from the same patient revealed variations in the transcriptional control elements (Loeber and Dorries, 1988). Transcriptional control elements regulate glial cell-specific expression and the extremely restricted host range of JCV (SinoHara et al, 1989; Kenney et al, 1984). Hence mutations or rearrangements in the JCV regulatory region may result in the spread of the virus from kidney, where it remains latent, to the brain, where it successfully grows.

Previous studies used in vitro binding assays to suggest that the NF1 binding motifs may play a role in the glial cell-

specificity of JCV (Khalili et al, 1988; Amemiya et al, 1989; Tamura et al, 1988). No functional assays were done. In order to conclusively determine the role of NF1 sites in glial cell-specific expression of JCV, I continued the preliminary studies initiated in our laboratory (Nakshatri et al 1990a). The main objectives of this chapter are:

1) To examine the functional role of NF1 binding sites in the glial cell-specific expression of JCV by performing site directed mutagenesis of NF1 motifs in the JCV regulatory region.

2) To correlate the in vivo activity of JCV promoter-enhancers harbouring WT and mutant NF1 sites with in vitro DNA-protein interaction, employing DNaseI footprinting assays.

3) To examine the interaction of factors with JCV promoter-enhancer by mobility shift assays.

4) To isolate the cDNA encoding the factor(s) interacting with the NF1 II/III site.

3.2 RESULTS

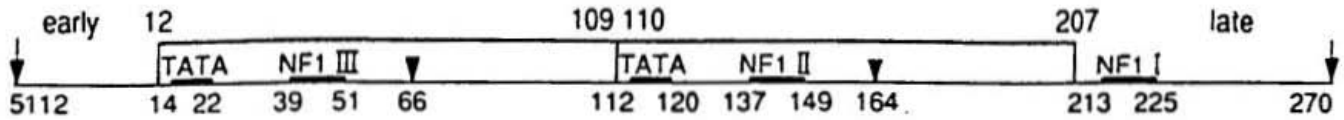
3.2.1 Role of specific NF1 sequences in *in vivo* activity of JCV enhancer

Previous studies had shown that efficient activity of JCV early regulatory region is restricted to cells of neural origin (Kenney et al, 1984; Nakshatri et al, 1990a). Using nuclear extracts from undifferentiated, DMSO, and RA-treated P19 cells for DNase I footprinting, it was shown that three completely protected regions, all containing NF1 binding motifs, were produced specifically for RA-differentiated cell extracts. Competition with oligonucleotides that span these protected regions suggested interaction of the same factor with all three sites (Nakshatri et al, 1990a).

I examined the functional role of NF1 motifs in RA-treated P19 cell-specific expression of JCV. Site-directed mutagenesis of the JCV regulatory region was undertaken. Several mutations were introduced into the three NF1 binding sites in the enhancer of JCV and the enhancer fragments containing each mutation were inserted into the reporter plasmid pSV0cat, a promoter-enhancer less vector (Fig. 3.1). Plasmids were then tested for their functional activity in undifferentiated (UD), DMSO (DM)- and RA-differentiated (RA) P19 cells. Mutation of NF1 and E4TF1 binding sites in DNase I protected region I (Fig. 3.1) did not result in significant alterations in CAT activity when tested in RA P19 cells

Fig. 3.1 Wild type and mutated NF1 motifs in JCV_E sequences in CAT plasmids and in DNaseI protected regions.

(Top) Diagram of sequences present in CAT plasmids. Arrows indicate the boundaries of sequences for the first seven constructs listed below, and arrowheads indicate sequences for the last two constructs listed below. Early and late sides are indicated. TATA boxes and NF1 site motifs are indicated and delineated by dark lines. Nucleotide positions are given below and above the lines. Boxes indicate the 98 bp repeats. (Bottom) List of mutated plasmids and mutated sequences and delineation of DNaseI protected sequences for the three NF1 sites. Mutated NF1 nucleotides are indicated by lower case letters. Wild-type NF1 nucleotides are indicated in $pJCV_Ecat$ by capital letters and in other plasmids by dashes. Inverted palindromic sequences are underlined for pJC_Ecat. DNaseI-protected sequences of Fig.3.4 are indicated by overlines and nucleotide numbers. The complete oligonucleotide sequence protected for regions NF1 II and NF1 III is 5′-AGGGATGGCTGCCAGCCAAGCATG-3′ and that for region NF1 I is 5′-GGAAGTGGAAAGCAGCCAAGGGAA-3′.



Plasmid	NF1 III	NF1 II	NF1 I
	34 57	132 155	208 231
pJCEcat (WT)	TGGCTGCCAGCCA	TGGCTGCCAGCCA	TGGAAAGCAGCCA
	34 57	132 155	
pJCEcat I	----------	----------	·a--------a·c
	36 45	134 143	208 231
pJCEcat II	----------	----------a·c	----------
	36 45	134 143	
pJCEcat I.II	----------	----------a·c	·a--------a·c
pJCEcat I.III	gta-------a·c	----------	·a--------a·c
			208 231
pJCEcat II.III	gta-------a·c	gta-------a·c	----------
pJCEcat I.II.III	gta-------a·c	gta-------a·c	·a--------a·c
pRIIJCEcat (WT)		----------	
pmRIIJCEcat		gta-------a·c	

(Fig.3.2, I). However, constructs which contained mutated GCCA nucleotides of the right half side of the perfectly palindromic NF1 region II motif (Fig. 3.1) had 3.6 (Fig. 3.2, II) and 3.1-fold (Fig. 3.2, I.II) lower cat activity in P19 RA cells. Surprisingly, these mutants had more than 2-fold higher activity in P19 DM muscle cells (Fig. 3.2, DMSO). The importance of combinations of the three JCV NF1 sequences was also examined with mutations in regions II plus III, I plus III and all the three sites (Fig. 3.1). These mutants were 5.8- 4.3- and 7-fold, respectively, lower in cat activity in P19 RA-differentiated glial cells (Fig. 3.2, II.III, I.III, I.II.III). Once again these mutants displayed more activity in P19 DM muscle cells compared to wild type (Fig. 3.2, DMSO). The results suggested that the NF1 binding sites located within, but not outside, the 98 bp repeats are important for the efficient expression of JCV early regulatory region in P19 RA-differentiated cells.

Next, the possibility that a single perfectly palindromic NF1 binding motif located in the repeats would be sufficient for glial cell-specific expression of JCV was examined. Plasmids with a single repeat region which had either a wild type or mutated NF1 site were constructed (Fig.3.1). Wild type region II plasmid (Fig. 3.2, pRII) exhibited a substantial amount of activity in P19 RA cells. This was similar to the activity seen for region I plus III

mutant (Fig. 3.2, I.III). The cell specificity of these plasmids was also similar to that observed for intact enhancer constructs. Mutation of the region II NF1 site alone (Fig. 3.1, pmRII) reduced the cat activity 3.5-fold in P19 RA cells (Fig. 3.2, pmRII) compared with wild type region II plasmid. The results suggested that the NF1 site located in the 98 bp repeats alone is sufficient for RA P19 cell-specificity of JCV early regulatory region. However, in P19 DM muscle cells, region II mutant (pmRII) showed 2.4-fold more activity compared to wild type pRII construct. Also, its activity was 1.9-fold more in P19 DMSO than in P19 RA cells (Fig. 3.2, compare pRII and pmRII). Thus, the results suggested that the mutations in NF1 site in 98 bp repeat resulted in slightly increased activity in P19 DM muscle cells.

3.2.2 Role of NF1 sequences in *in vitro* activity of JCV enhancer

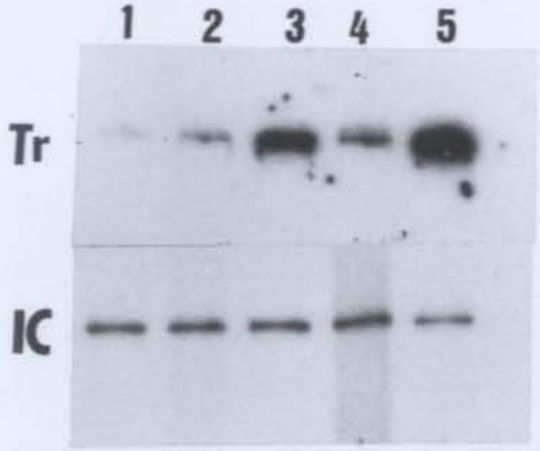
The effect of NF1 mutations within the 98 bp repeats on the expression of JCV early control region was examined by *in vitro* transcription assays. Substantial activity was observed for neural cell extracts (Fig. 3.3, left panel, lanes 3 and 5). Negligible activity was observed in undifferentiated cell extracts (Fig. 3.3 left panel, lane 1). Template with mutations of the three NF1 sites greatly reduced the levels of transcript (Fig.3.3, left panel, lanes 2 and 4). Template containing mutations in region I NF1 motif supported wild type

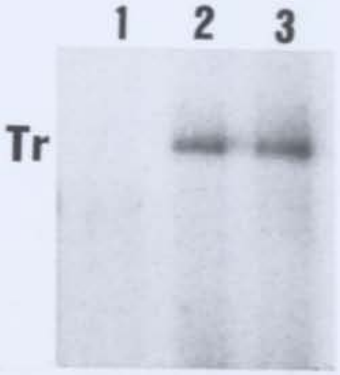
Fig. 3.2. Effect of mutation of JCV NF1 sites on <u>in vivo</u> activity.

Results are for CAT assays in undifferentiated (UD), dimethylsulfoxide (DMSO)- and retinoic acid (RA)-differentiated P19 embryonal carcinoma cells. NF1 site mutations are indicated by the unique letters for the plasmids of Fig. 3.1. The cells were transfected, 8 hours after being plated, with 10 μg of test plasmid and 10 μg of pUC19 DNA. Values for CAT activity were calculated from assays with less than 30% conversion and were normalized with assays for Rous sarcoma virus promoter-driven β-galactosidase plasmid. Results are averages of three experiments. The error bars in the figure represent standard deviations.

mRII
RII
I.II.III
I.III
II.III
I.II
II
I
WT
0
20
40
60
80
% Conversion
RA
DMSO
UD

Fig. 3.3 Requirement of JCV NF1 sites for *in vitro* activity. *In vitro* transcription assay products were resolved in 5% acrylamide gels containing molecular weight markers. (Left) DNA templates were digested with NcoI. Lanes:1, pJCEcat and 75 μg of extracts from P19 UD cells; 2 and 4, pJCEcat I.II.III and 60 and 75 μg, respectively, of extracts from RA-differentiated cells; 3 and 5, pJCEcat and 60 and 75 μg, respectively, of extracts from RA-differentiated P19 cells. Tr, 550 nt transcript; IC, 286 bp end-labelled fragment serving as internal control which was added before phenol extractions. (Right) Extracts were 35 μg of RA-differentiated glial cells and DNA templates were digested with PvuII. Lanes: 1, pJCEcat and incubation in the presence of 1 μg of alpha-amanitin RNA polymerase II inhibitor per ml; 2, pJCEcat; 3, pJCEcatI. Tr, 162 nt transcript. The autoradiographs presented in right and left panels are from different experiments. The results were consistent for two experiments.


1
2
3
4
5
Tr
IC


1
2
3
Tr

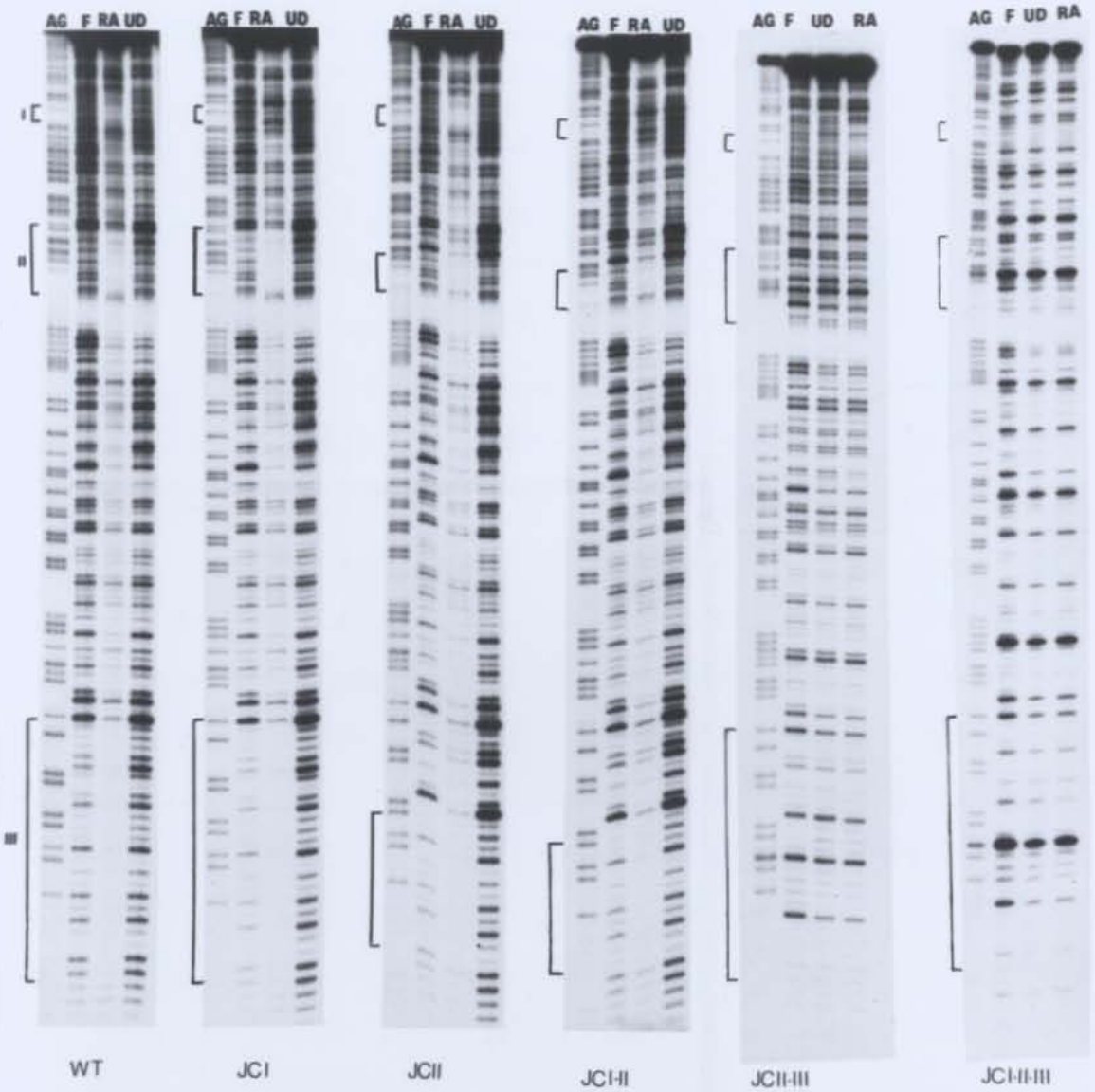
levels of *in vitro* transcription (Fig. 3.3, right panel). Transcription was by RNA polymerase II since alpha-amanitin at 1 μg/ml concentration blocked all transcriptional activities (Fig. 3.3, right panel). Thus, these results supported the *in vivo* observations and further indicated that the NF1 sites within the two 98 bp repeats of JCV, but not the region I site, are essential for efficient functional activity of JCV enhancer in glial cells.

3.2.3 Effect of mutations in NF1 binding sites on DNA-protein interactions

Since mutating one or both palindromic NF1 sites within the 98 bp repeats greatly reduced JC enhancer activity, I also examined *in vitro* DNA-protein interactions in the context of mutated NF1 sites. For this purpose, DNaseI protection assays were carried out. The region I mutation eliminated protection, but only for region I (Fig. 3.4, JCI). For the mutation of the GCCA half side of NF1 motif of region II, protection was restricted to the central nt of the region protected for wild type probe and excluded the GCCA sequences (Fig. 3.1 and Fig. 3.4, JCII and I.II). In addition, there was a similar effect on the DNaseI protection of the non-mutated region III (see the region III for lane RA of JCII). The double mutation (JCI.II) displayed a simple additive effect (Fig 3.4, JCI.II). Probes containing mutations in NF1 binding motifs of both half sides for regions II and III mutations were completely

Fig. 3.4 Effect of mutations on DNaseI footprinting of JCV NF1 sites.

The protection from DNaseI by extracts is visualized as fainter regions. NF1 sites mutated in the probes are indicated at the bottom and correspond to those of Fig. 3.1. For probes, nt 5112 to 270 of JCV wild-type and mutated fragments (Fig. 3.1) were cloned into the pUC19 XbaI site, and Sal I- Sma I fragments were end-labelled only on the bottom Sal I end with [alpha-^{32}P]dCTP. Lanes: AG, chemical cleavage of purines; F, free DNA of assay with no nuclear extract; UD, undifferentiated cell extract; RA, retinoic acid-differentiated cell extract. Protected regions I,II, and III are bracketed and indicated. Protected sequences and binding motifs for transcription factors are as delineated and described in the legend to Fig. 3.1.


AG F RA UD
I
II
III
WT
JCI
JCII
JCI-II
AG F UD RA
JCII-III
JCI-II-III

unprotected in regions II and III (Fig. 3.4, JCII.III). Mutations in both half sides of all three NF1 binding sites abolished protection in all three regions (Fig. 3.4, JCI.II.III).

3.2.4 Nature of proteins interacting with NF1 sites

To identify the cellular factors from P19 cells that interact with NF1 binding sites of JCV regulatory region, DNA-protein binding was further evaluated by mobility shift assays. First JCV whole enhancer was used as a probe in mobility shift assays. In addition to complexes observed for UD cell extracts, a low mobility DNA-protein complex was detected only with extracts from P19 RA cells (Fig. 3.5, arrow). The amount of this complex increased with 2-fold higher protein concentration for P19 RA cell extracts, while the amounts of other complexes did not change for UD and RA extracts (Fig. 3.5, compare lanes UD and RA). The results suggested that a factor restricted to neural cells specifically interacts with JCV enhancer and its affinity to JCV enhancer is also higher.

Since the NF1 II/III located in the repeats was found to be important for <u>in vivo</u> activity in RA P19 cells, DNA-protein binding was also examined by mobility shift assays using an oligonucleotide for the region II NF1 motif as a probe. A diffuse DNA-protein band was detected with P19 RA cell extracts but not with UD cell extracts (Fig. 3.6 UD and

Fig. 3.5. In vitro binding of glial cell transcription factor(s) with JCV promoter-enhancer in mobility shift assays. Lanes: F, no nuclear extract; UD, undifferentiated cell extract; RA, retinoic acid-differentiated cell extract. Assays were done using the intact JCV enhancer probe described in Fig. 3.4 for wild type. Left and right panels, 2.5 and 5 μg, respectively, of protein per assay. Arrow indicates the specific low-mobility DNA-protein complex for RA lanes. Arrowhead indicates free probe not bound to factor(s).

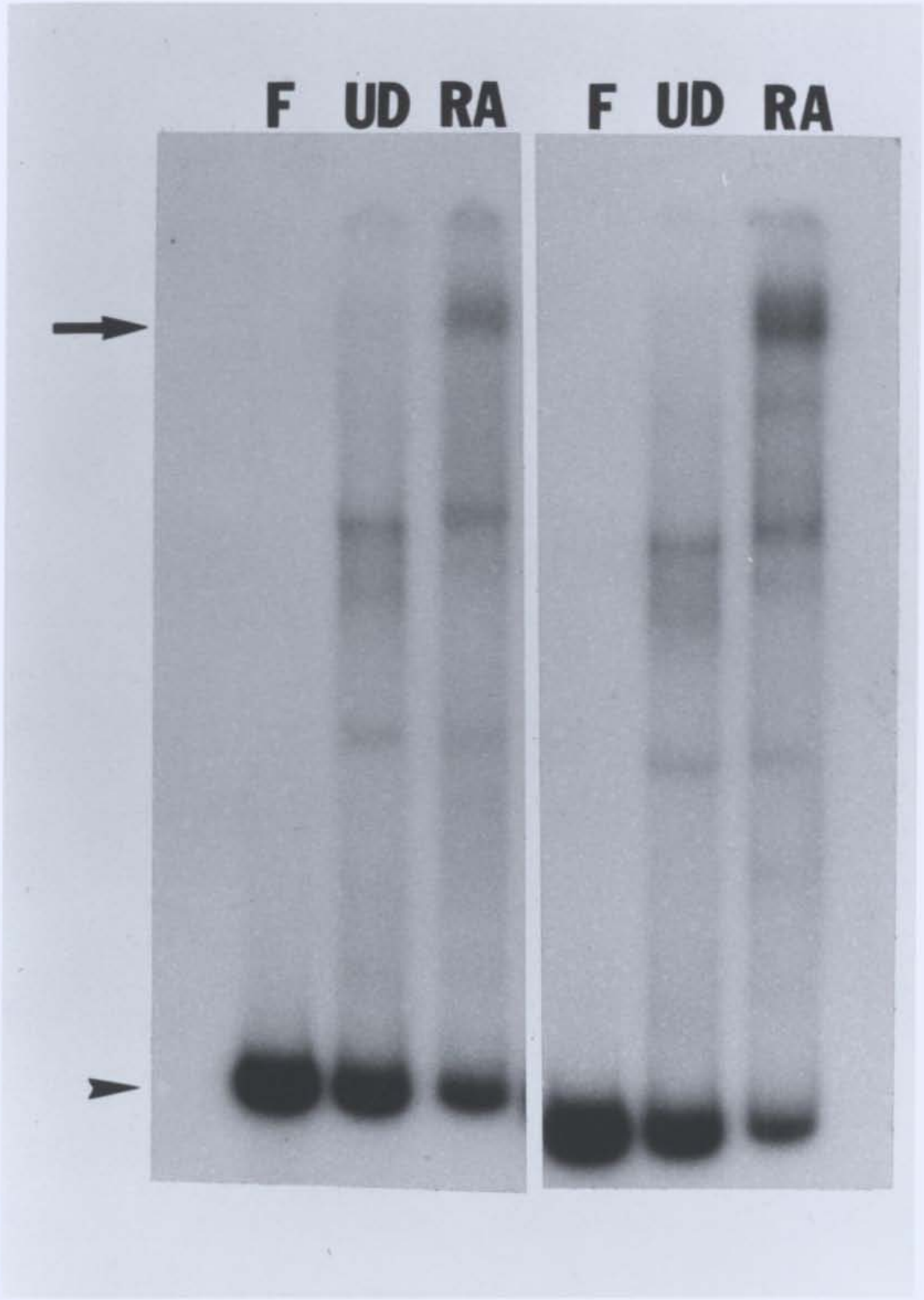
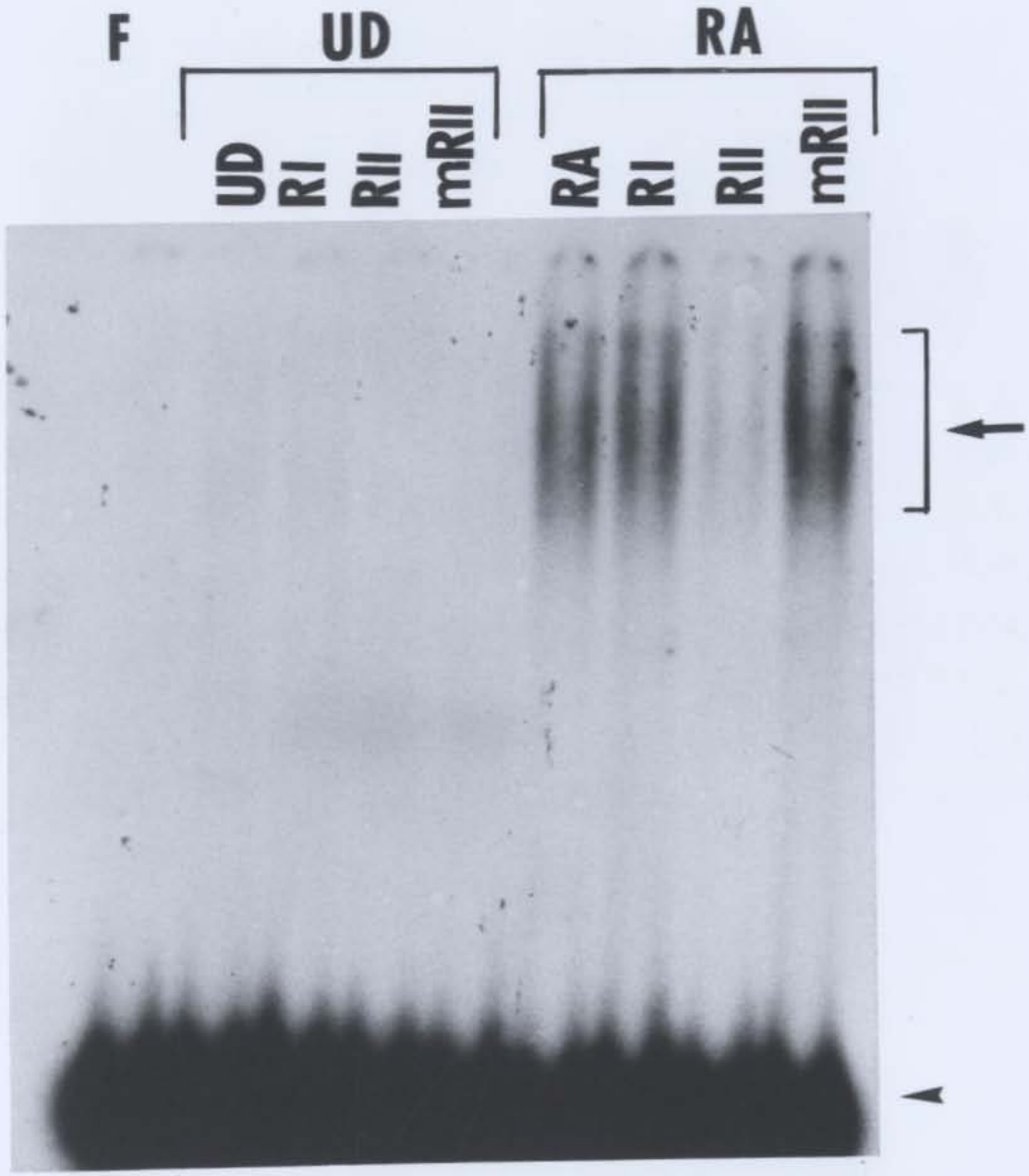

F UD RA
F UD RA

Fig.3.6 In vitro binding of glial cell extract with NF1 II/III oligonucleotide using mobility shift assays.

Assays were done using end-labelled double stranded NF1 II/III oligonucleotide sequence as a probe and 5 μg of protein in extracts. RI, RII, and mRII indicate competition with NF1 I, NF1 II/III and mutant NF1 II/III, respectively, oligonucleotides in 200-fold excess amounts. The sequences of wild type RII oligonucleotide used as probe and the oligonucleotide competition were as follows: RI, 5'-AAGGGGAAGTGGAAAGCAGCCAA-3'; RII, 5'-TGGCTGCCAGCCAA-3'; mRII, 5'-GTACTGCCAGACCAGCA-3'. The arrow indicates free probe and the arrowhead specific low-mobility DNA-protein complex.


F
UD
RA
UD
RI
RII
mRII
RA
RI
RII
mRII

RA). Moreover, competition with wild type but not mutant NF1II/III oligonucleotide abolished complex formation (Fig. 3.6, RII and mRII). Interestingly, competition with oligonucleotide corresponding to the protected region I did not abolish the complex formation (Fig. 3.6, RI). The results suggested that factor(s) restricted to P19 glial (RA) cells but not UD cells interact specifically with the NF1 II/III sequence of JCV enhancer. The results also provided a clue that the protein factor(s) interacting with NF1 I and NF1 II/III are different since NF1 I sequence could not compete with the factor interacting with NF1 II/III.

3.2.5 Isolation and preliminary characterization of a cDNA encoding the factor binding to NF1 II/III binding motif of JCV

3.2.5.1 Screening of the P19 RA library

The site-directed mutagenesis studies clearly suggested that NF1 II/III binding motif plays an important role in P19 RA cell-specific expression of JCV. In addition, the in vitro binding assays indicated the interaction of P19 RA cell-specific transcription factor(s) with the NF1 II/III oligonucleotide. Hence, it was of interest to isolate a cDNA that encodes for a protein able to bind the NF1 II/III sequence from retinoic acid-differentiated P19 cells .

Sequence-specific DNA binding proteins can be purified by DNA affinity chromatography (Chodosh et al, 1986). However, this requires large amounts of starting material. Therefore,

I decided to isolate the cDNA encoding for the factor interacting with NF1 II/III by screening an expression library prepared from RA-differentiated P19 cells using Southwestern blot analysis. This technique was first used to isolate a cDNA encoding for the immunoglobulin enhancer binding protein (Singh et al, 1988). The technique involves direct screening of an expression library with a specific DNA sequence as a ligand.

Approximately $1x10^6$ plaques from P19 RA λ gt 22A expression library were screened by Southwestern blot analysis as described in 2.2.13. Primary screening resulted in isolation of two positive clones (Fig. 3.7). To further confirm specificity, one of the clones (λ K1) was subjected to further testing. The recombinant phage from this clone was plated and replica filters were exposed to nick-translated wild type NF1 II/III, mutant NF1 II/III, and non-specific NF1 I oligonucleotides. Wild type NF1 II/III showed positive plaques but not mutant or NF1 I oligonucleotides (Fig.3.8 compare A and C with B and D, respectively). The results suggested that the isolated cDNA encodes a factor that binds to NF1 II/III in a sequence specific manner.

To determine whether the isolated cDNA encodes a factor which is restricted to RA P19 cells, Northern blot analysis of total RNA isolated from UD and RA-differentiated P19 cells was undertaken. The cDNA from λ K1 recombinant phage

Fig. 3.7 Identification of λ phage recombinant cDNA clone which specifically binds to NF1 II/III element.

The two positive clones were detected by oligonucleotide probe in the Southwestern blots. The positive cDNA clones detected after primary screening are indicated by arrowheads.

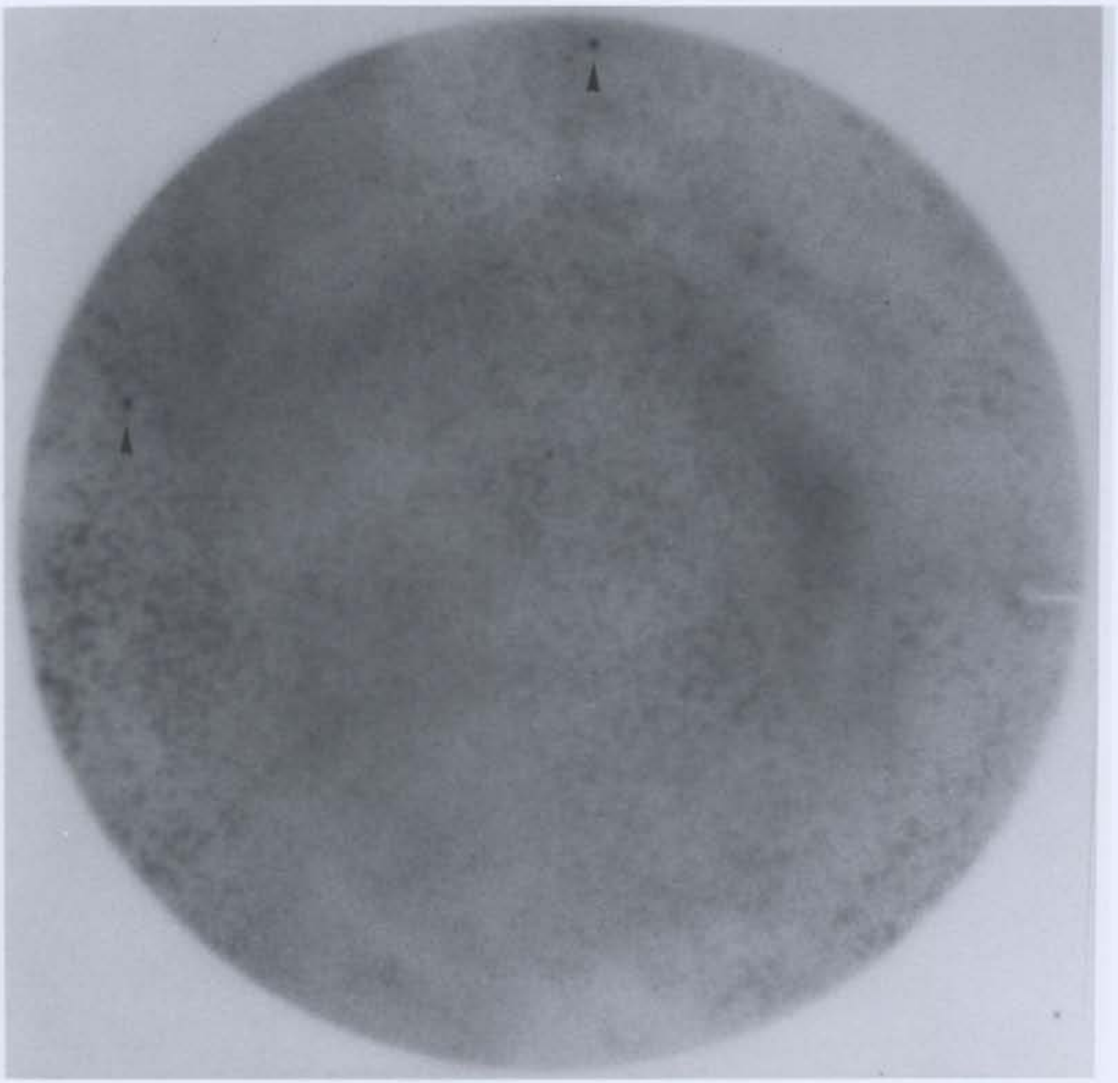
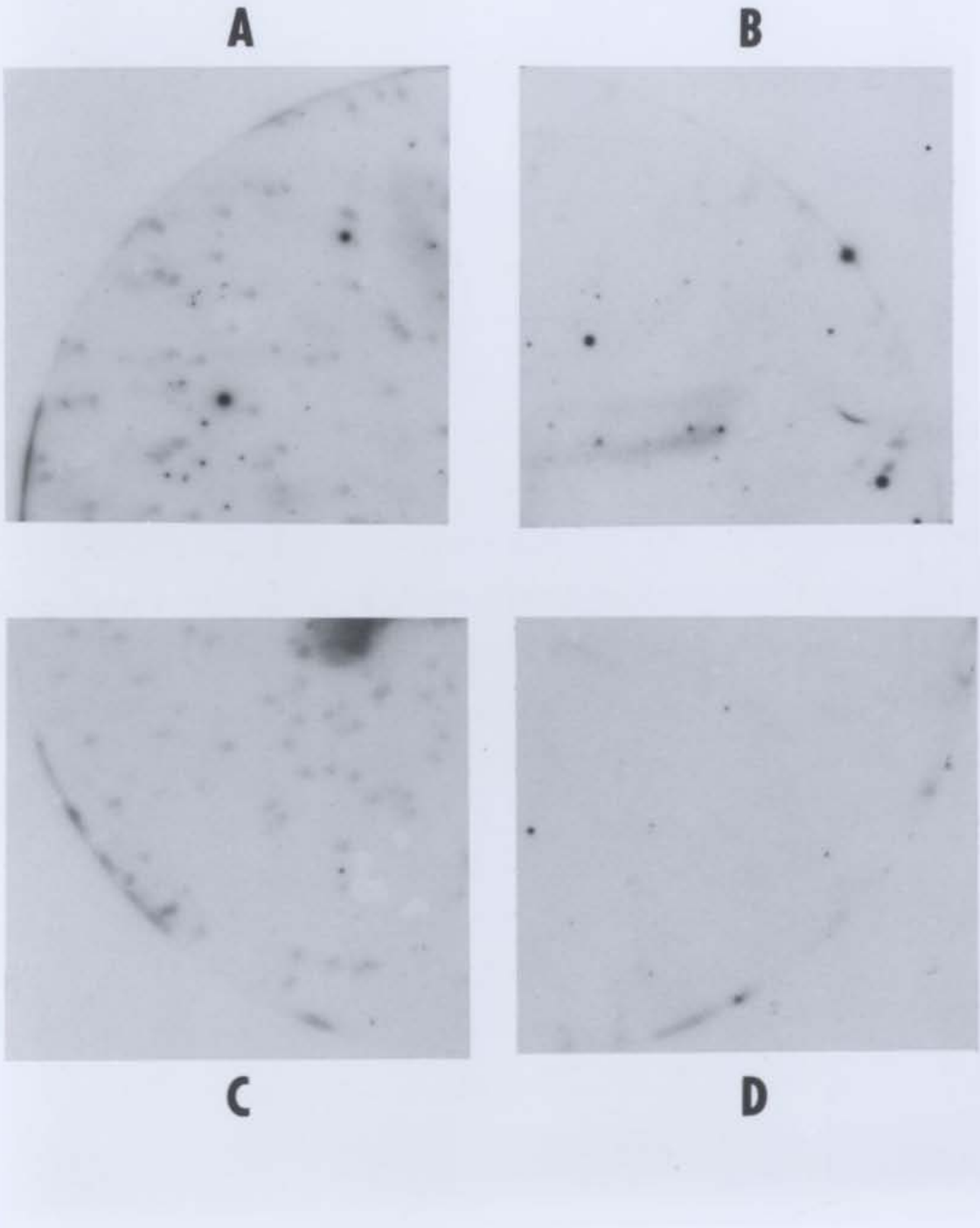


Fig. 3.8 Specificity for NF1 II/III binding by the positive recombinant cDNA clone, λ K1.

The recombinant K1λ phage was plated and replica filters were screened in Southwestern blots as in Fig. 3.7 with wild type NF1 II/III oligonucleotide in panels A and C, mutant NF1 II/III oligonucleotide in panel B, and NF1 I oligonucleotide in panel D. Only quarter filters are shown for comparison.


A
B
C
D

was used as a probe. A message of approximately 1.2 kilobase pairs (kb) was detected for RNA from P19 RA cells and very low level of message in UD cells (Fig. 3.9). The results indicated that the factor isolated is restricted to P19 RA cells. Taken together, the isolated cDNA encodes a P19 RA cell factor that specifically interacts with the NF1 II/III binding site located in the 98 bp repeats which was shown important for glial cell-specific expression of JCV.

3.2.5.2 Transcriptional activity of cDNA in nonglial cells

To examine the transcriptional activity of the cDNA and to find out its ability to complement for the expression of JCV regulatory region in non-neural cells, the cDNA was cloned in eucaryotic expression vector pCMV. The recombinant plasmid, pCMVNF1 was cotransfected with JCV_Ecat and JCV_Lcat plasmids into HeLa cells. In the absence of pCMVNF1, none of the CAT reporter plasmids showed activity, confirming their glial cell-specificity. However, cotransfection with pCMVNF1, $pJCV_E$ and $pJCV_L$ CAT constructs showed 3.5- and 4.0-fold activity, respectively, in HeLa cells (Fig. 3.10). The expression of pCMV NF1 had no effect on the expression of BK virus regulatory region consisting of NF1 sites. This suggests that the isolated cDNA is functional and complements for the activity of JCV control region in nonglial cells.

Fig. 3.9 Northern blot analysis of P19 total RNA, using K1 cDNA as probe.

The RNA isolated from UD and RA-differentiated P19 cells were resolved on 1% formaldehyde-containing agarose gels. A northern transfer of this RNA was probed with the isolated cDNA fragment from λ K1 phage clone. RNA molecular weight markers are indicated in kb on right side.

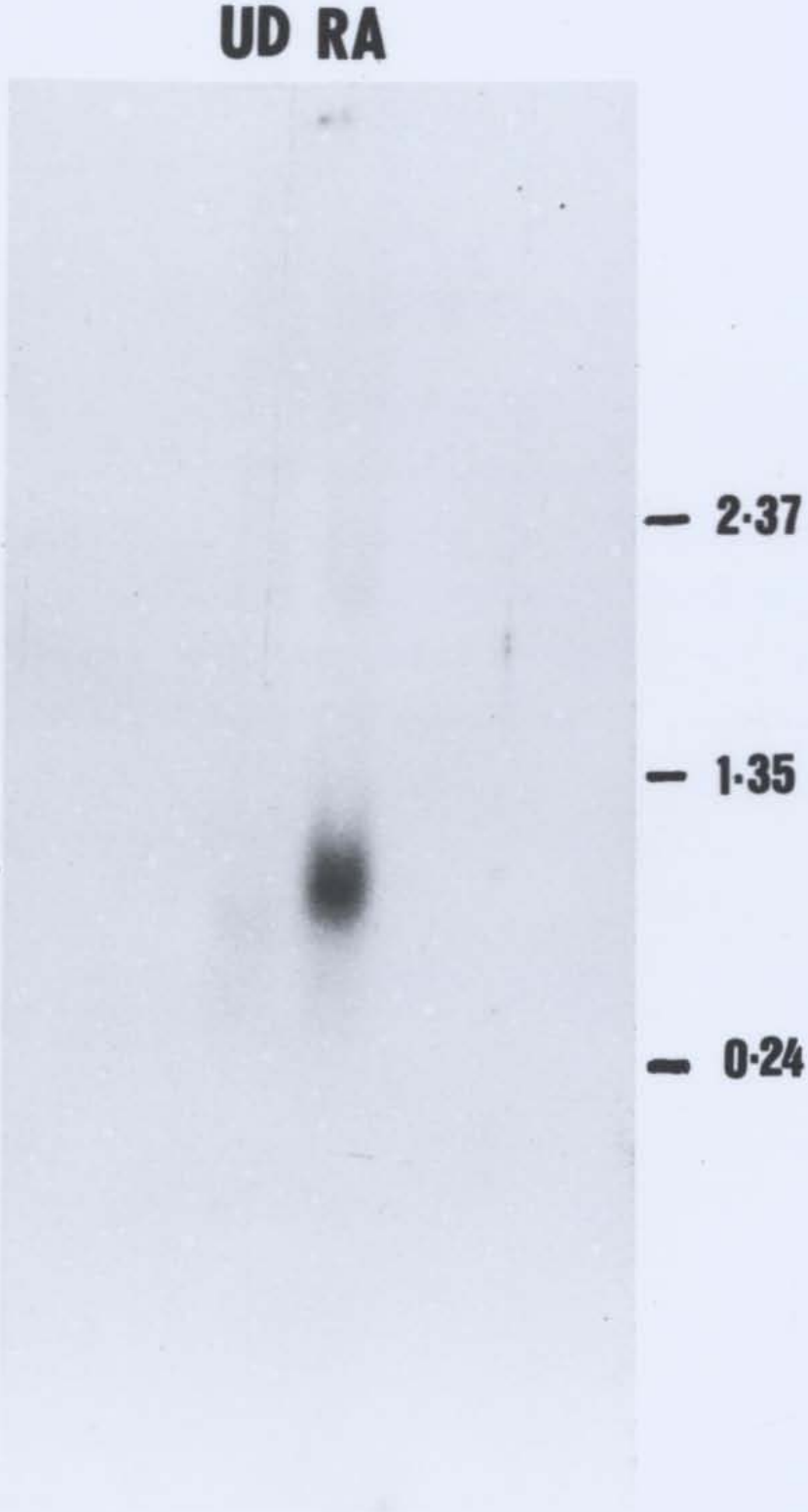
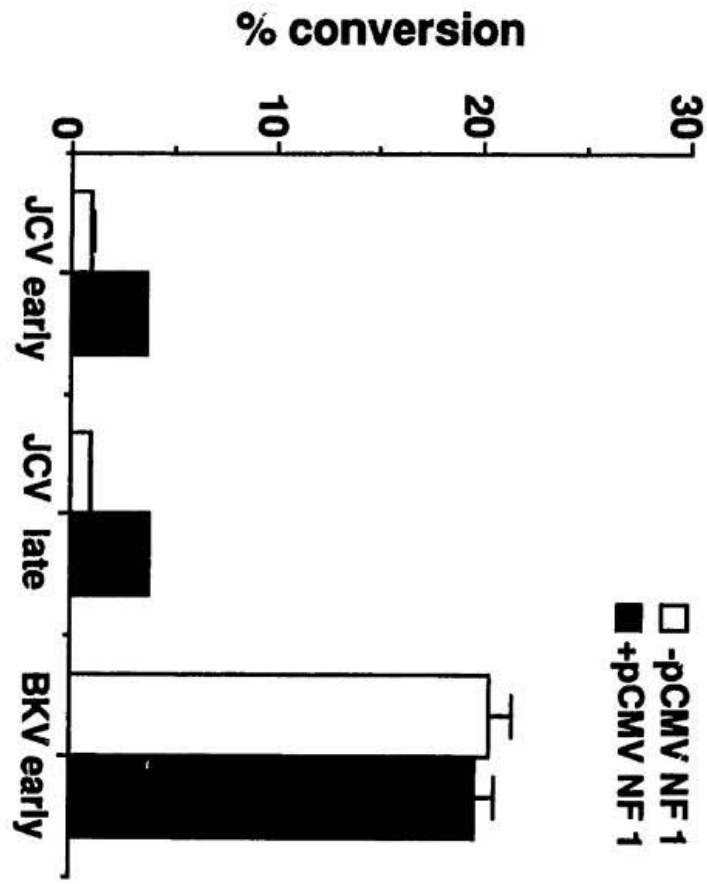

UD RA
2·37
1·35
0·24

Fig. 3.10 Expression of JCV early, late and BKV early promoters in nonglial cells in response to clone K1NF1.

The lanes for JCV early, JCV late and BKV early promoters are indicated. The assays were with and without cDNA, the pCMV expression vector for K1NF1. Ten μg of reporter plasmids were cotransfected with 10 μg of pCMV expression vector for K1NF1 into HeLa cells. Results are averages of two different experiments.


% conversion
0
10
20
30
JCV early
JCV late
BKV early
-pCMV NF 1
+pCMV NF 1

3.3 DISCUSSION

The purpose of the present study was to determine the functional role of NF1 binding motifs in the expression of the JCV early promoter-enhancer. Site-directed mutagenesis of these motifs led to the novel finding that the NF1 motif inside, but not outside, the 98 bp repeats (Fig. 3.2, II.III and I.II.III) is important for RA P19 cell-specific expression of JCV. This observation provided the basis for further studies such as isolation and preliminary characterization of the factor interacting with NF1 II/III described in this chapter. The objectives of the study were achieved, largely through site-directed mutagenesis and cDNA cloning.

Mutation of one of the perfectly palindromic NF1 motifs present in the 98 bp repeat resulted in a 3.5- to 4-fold reduction and mutation of both motifs resulted in 7-fold decrease in activity, specifically in RA P19 cells (Fig. 3.2). However, the mutation of the NF1 region I motif on the late side of 98 bp repeats had no significant effect. The <u>in vivo</u> observations were further confirmed by <u>in vitro</u> transcription assays (Fig. 3.3). These data provide direct experimental evidence that the NF1 factors interacting with these sequences are important in RA P19 cell-specific expression of JCV.

Using cell extracts from retinoic acid-differentiated P19 cells in DNaseI protection assays, it was shown previously that in addition to the protection of three NF1 binding sites

in JCV enhancer, E4TF1, overlapping the NF1 I was also protected (Nakshatri et al, 1990a). The functional role for this factor in glial cell-specificity remained unclear. In the present study, the mutations introduced into the NF1 I also disrupted the binding site for the factor interacting with this motif, as revealed by DNaseI protection assays (Fig. 3.1 and Fig. 3.4, JCI). Since the mutations in NF1 I had no effect on the expression of JCV_E, this suggests that the E4TF1 motif has no functional role in glial cell-specific expression of JCV.

To examine the role of the individual NF1 motifs in the 98 bp repeat, wild type (WT) and mutated single 98 bp repeats were tested for comparison of their functional activity in UD, DM, and RA P19 cells (Fig. 3.2, RII and mRII). Three observations could be made. 1) For RA P19 cells there was 3.5-fold lower activity as compared to whole enhancer construct for pRII. This lower level of activity was similar to the level seen when one of the two repeat region sites of the whole enhancer construct was mutated. The implication is that region I NF1 has no major role in glial cell-specificity and region II activity is the same in I.III mutant and pRII. 2) The 3.5-fold decrease in activity after mutating the repeat region NF1 II site was similar to the results obtained with mutated complete enhancer constructs. This demonstrated that effects of such mutations were retained for a single site. 3)

There was a muscle cell-specific increase in activity when the single NF1 site was mutated. This observation is consistent with the results for the different mutations of repeat region NF1 sites within the context of whole enhancer.

In an earlier study (Tada et al, 1989), the oligonucleotides encompassing NF1 I, NF1 II/III, and the pyrimidine rich region and the TATA box of JCV regulatory region were placed in front of the SV40 promoter in pA10cat. The activity of these constructs was assessed in glial and nonglial cells. These constructs showed activity in glial cells. However, the constructs containing NF1 I and TATA box alone showed activity, even in nonglial cells, although not to the same extent as that observed in glial cells. Interestingly, the construct harbouring the NF1 II/III sequence failed to display any detectable activity in nonglial cells while the vector, pA10 cat, by itself showed basal level of activity. The authors interpreted the results as suggesting that the NF1 II/III sequences suppressed the SV40 promoter activity and that these sequences are positive and negative regulators of expression in glial and nonglial cells, respectively (Tada et al, 1989). My observation that the NF1 II/III mutants exhibit higher activity in DMSO P19 cells give credence to these suggestions. More importantly, while Tada et al (1989) analyzed the effect of NF1 sequences using a heterologous promoter, in my studies the functional role of

NF1 sites in the glial and nonglial expression of JCV was assessed in the context of the JCV promoter. Since the activity of JCV promoter-enhancer in DMSO-differentiated P19 cells is low, the increased activity of NF1 mutants in these cells compared to WT can be considered significant. Compared to the regulatory regions of BKV and SV40, the activity of JCV promoter-enhancer in P19 RA-differentiated cells is lower by several-fold (Nakshtri et al, 1990a). Hence the reduced activity of NF1 mutants in RA-differentiated P19 cells, although modest, is significant.

JC-HEK, a JC virus adapted to growth in human embryonic kidney cells (HEK), displayed rearrangements in the control region for early transcription. The control region of JC-HEK, when placed in front of CAT gene in pSV0cat vector exhibited activity similar to that of SV40. It also displayed 4 times more activity than that of prototype MAD1 JCV strain in HEK cells (SinoHara et al, 1989). This again suggests that the rearrangements in the control region might have resulted in alterations in the potential NF1 II/III sequences and thus permitted the expression of JCV early promoter-enhancer in nonglial cells such as HEK cells. These results are qualitatively consistent with the results observed for in vivo activities of pRII and pmRII in DMSO-differentiated P19 cells.

The results of experiments using CAT constructs with a single 98 bp repeat confirmed that, while the duplicated

repeats with the two functional palindromic NF1 sites have more activity than constructs with a single NF1 site, NF1 site of one repeat can function independently for glial cell-specific expression. Furthermore, the tissue specificity of the perfectly palindromic NF1 sites appear to be involved in both the activation of JCV expression in glial cells as well as the inhibition of expression in nonglial cells. Previous studies have shown that a protein of 56 kDa binds to the penta nucleotide repeat sequence, AGGGAAGGGA adjacent to NF1 II and III and prevents the expression of JCV_E in nonglial cells (Sharma and Kumar, 1991). Therefore, it appears that the glial cell-specific expression of JCV is tightly regulated by *cis*-acting elements that act positively in glial cells and negatively in nonglial cells. This is reminiscent of regulation of the neuron-specific expression of growth associated protein, SCG10, by *cis*-acting negative regulatory element (Mori *et al*, 1990). Possibly, the presence of two perfect palindromic NF1 sites in the repeats act indirectly to overcome the effects of repressors interacting with pentanucleotide repeat sequences and allow transcription from JCV_E.

P19 cells have proven to be a good system to demonstrate this mode of tissue-specific regulation of gene expression, as they can be differentiated into different cell types from an identical genotype. Hence, I could demonstrate

the importance, specifically, of the NF1 binding site present in the 98 bp repeat in glial and nonglial cell-specific expression of the JCV early promoter. However, a heterogeneous population of neuronal cells are induced by RA treatment of P19 EC cells. Hence, this may affect interpretation of my data. For example, the JCV expression is regulated positively in glial cells and negatively in nonglial cells. In in vitro transcription results the efficient activity of JCV_E noticed could be due to positive regulators from glial cells in a mixed population of neural cells (Fig. 3.3). The negative regulators from other neural cells might have an effect on JCV expression. Depending upon relative concentrations and affinities of gene-specific regulators, the expression patterns could alter.

There has been a lot of discrepancy in studies reporting the start sites for the early and late transcription of JCV (Daniel and Frisque, 1993). In the present study the importance of NF1 binding site in glial cell-specific JCV early transcription was confirmed in vitro by performing in vitro transcription assays (Fig. 3.3). The results suggested that the transcription is initiated from position 5115-5125 utilizing the TATA box situated near the origin in repeat A (Fig. 1.1). This result is consistent with the observations reported recently by Daniel and Frisque (1993).

The effect of in vivo activity of NF1 binding site

mutations was correlated with alterations in the *in vitro* interactions with cell specific factors, as assayed by DNaseI footprinting (Fig. 3.4). Interestingly, the mutation in the NF1 binding site in the central 98 bp repeat abolished protection in the NF1 binding site present in both repeats, suggesting the cooperative interaction of the proteins binding to these sites (Fig. 2.4 JCII). This was consistent with the lesser effect on activity of mutating the second of the two repeat region sites. This applies even when the second site is the down stream site, which appears to have a greater effect (Fig. 3.2, compare WT and II versus I.III and I.II.III effects, respectively).

Earlier studies had indicated that both NF1 I and NF1 II/III oligonucleotides competed with all the three NF1 sites in DNaseI protection assays (Nakshatri *et al*, 1990a). Hence, it was concluded that the proteins interacting with all the three NF1 sites of JCV may be the same and cooperative binding of the factor interacting with NF1 I and II was suggested. In addition, a report by Amemiya *et al* (1989) suggested that the proteins interacting with NF1 I and II sites are the same. However, in the present study, DNaseI protection assays with NF1 mutants clearly showed cooperative interaction of proteins binding to NF1 II and NF1 III but not to NF1 I and NF1 II (Fig. 3.4, compare JCI with JCI.II). Further, the results of competition experiments in gel shift assays with NF1 II/III

oligonucleotide showed that the factors interacting with NF1 I and II are different (Fig. 3.6). The difference in the results of the present studies could be due to the more sensitive techniques I used in assessing DNA-protein interactions.

NF1 binding sites have been found in several genes such as BK virus control region (Nakshatri et al, 1991), human papillomaviruses (Nakshatri et al, 1990b), myelin basic protein (MBP) gene of the central nervous system (Aoyoma et al, 1990), cytomegalovirus (Nowock et al, 1985; Jeang et al, 1987), flanking regions of chicken lysozyme gene (Borgmeyer et al, 1984), human IgM gene (Hennighausen et al, 1985), c-myc gene (Siebenlist et al, 1984) and several other genes of the CNS (Amemiya et al, 1992). The NF1 binding sites located in all these genes indicated that the minimum nucleotide (nt) sequence required for the binding of NF1 is 15-16 nt with a consensus sequence of $TGG(N)_6GCCAA$. However, the first three nt, TGG and the 6 nt spacer are sufficient for NF1 binding but an A or C nt following TGG increases the binding of NF1 (de Vries et al, 1985; Leegwater et al, 1985). Mutation of GCCA in the NF1 binding site located in the promoter of the immediate early gene of cytomegalovirus abolished the binding of the protein (Hennighausen and Fleckenstein, 1986). However, in the present study, mutations introduced into the half side of the NF1 II palindrome (Fig. 3.4, JCII and JCI.II) abolished the

binding for that region only and the other half side was still protected. Mutations in the both half sites completely abolished the binding of protein (Fig. 3.4 JC II.III, I.II.III). Since this sequence is a perfect palindrome which has pronounced two-fold rotational symmetry, possibly the protein binds as a dimer. This is reminiscent of the repressor-activator-operator interactions commonly observed in prokaryotes (Ptashne, 1992). Previously, NF1 was shown to form a dimeric complex with its binding site (Hennighausen et al, 1985). The perfect palindromic sequence of JCV NF1 II/III, but not the imperfect NF1 I sequence, may allow high affinity binding by protein dimers on DNA double strands.

Interestingly, while the sequence TGGGCGGCGGCCAA in pBR322 and the sequence TGGCAACTTGCCAA in a middle repetitive element of mouse DNA contain consensus NF1 binding motifs, they were shown to be nonbinders (Hennighausen et al, 1985). The presence of a G-C within the central non consensus region of the NF1 site in pBR322 disrupted the binding of NF1 protein. This suggests that the spacer sequence between the consensus regions is also important. The NF1 II/III sequence of JCV, including the spacer sequence is a perfect palindrome, while the NF1 sequences located in several other genes are non-palindromic except for the sequence TGG(N_6)CCA. This raises the possibility that the presence of the perfect palindrome sequence in JCV may impart additional advantage in

terms of binding affinity. This is consistent with the observation that a synthetic NF1 oligonucleotides having the perfect palindromic TGGCACTGTGCCA sequence exhibited 10-fold higher affinity than the NF1 oligonucleotides that did not have the pronounced 2-fold axis of symmetry, such as the NF1 site of IgH gene (Hennighausen and Fleckenstein, 1986).

NF1 is a large family of proteins and several forms are generated by alternative splicing (Santoro et al, 1988). There are tissue-specific NF1 proteins that allow the expression of genes in a restricted manner and existence of epithelial cell-specific NF1 factors that interact with the binding sites in the HPV 16 genome has been recently reported (Apt et al, 1993). Interestingly, preliminary studies from our laboratory have revealed the expression of both HPV 16 and 11, which contain multiple NF1 sites, in RA-differentiated P19 cells (Kasinadhuni, 1994). Whether the same NF1 protein is important for the activity of both JCV and HPV remains to be investigated. My construction of a cDNA library and the isolation of a candidate cDNA from this library could readily be used for this study. The RA- and DMSO-differentiated P19 cells also allow expression of BK virus containing multiple NF1 sites. However, while the expression of JC virus is restricted to RA-differentiated P19 glial cells, expression of BKV is observed in nonglial cells (Nakshatri et al, 1991). This may suggest the presence of different cellular NF1

proteins important for the expression of the two viruses. The interaction of glial cell-specific NF1 is essential for the restricted glial cell specificity of JCV.

The isolation of cDNA from an expression library prepared from RNA from RA P19 cells confirmed the presence of a cell-specific factor and its possible involvement in regulating the activity of JCV enhancer in RA P19 cells. Northern blot analysis revealed the RA P19 cell-specific expression of the cDNA (Fig. 3.9). Moreover, the binding specificity of this cDNA was revealed by its lack of interaction with mutant and non-specific oligonucleotides (Fig. 3.8). The expression of this cDNA in HeLa cells enhanced the activity of JCV early and late promoter-enhancers. In addition, it has no effect on the expression of the early promoter of another polyoma virus, BKV, which contains multiple NF1 binding sites in its regulatory region (Fig. 3.10). However, there is no evidence presented here that transactivation in HeLa cells requires the integrity of any of the NF1II\III sites. Co-transfection of pCMVNF1 with mutated promoter constructs should provide further information on this. It is interesting to note that the cDNA isolated previously from brain cells encoded a factor binding to the NF1 II/III site and activated the late but not early JC promoter in nonglial cells (Kerr and Khalili, 1991). The cDNA which I isolated encodes a factor that stimulates the activity

of both JCV_E and JCV_L, suggesting that the NF1 II/III binding site is bidirectional in function and that this site is important for expression of both promoters as will be discussed later (chapter 4).

Previous studies had shown that a protein of 45 kDa purified from calf brain supported the expression of JCV_E in HeLa cells in an in vitro transcription assay (Ahmed et al, 1990). A cDNA was subsequently isolated by screening an expression library prepared from human brain cells using NF1 II/III binding sequence as probe. Surprisingly, this cDNA encoded a 45 kDa protein which stimulated the late promoter and to a very limited extent the early promoter (Kerr and Khalili, 1991). These results, although reported from the same laboratory, are inconclusive. However, from the present study it is clear that different factors are involved in the activation of JCV early and late promoters. The following observations prompt me to suggest that the cDNA isolated by me is different from that isolated earlier (Kerr and Khalili, 1991). 1) My studies used a mouse cell line whereas the other study used human brain cells. 2) It is possible that there may be multiple factors that regulate the early and late gene expression or the same factor may affect both early and late JCV gene expression. This is consistent with the observation that the cDNA I isolated supported the expression of early and late genes equally, while the cDNA isolated by Kerr and

Khalili (1991) activated only the late promoter. 3) NF1 is a family of proteins and hence different species of NF1 might exist which play a role in gene expression as reported earlier (Santoro et al, 1988).

From the preliminary study presented here, it is not clear whether the cDNA encodes NF1. However, the cDNA encodes a factor that interacts with the NF1 II/III motif of JCV in vitro and activates the JCV promoter-enhancer in vivo. Clearly, further studies such as sequence analysis of cDNA and in vitro binding studies with the isolated cDNA are needed to characterize the nature of this factor. At present, experiments on the detailed characterization of this factor are being undertaken by another graduate student in our laboratory.

NF1/CTF is involved in activating transcription and also replication of adenovirus DNA (Jones et al, 1987). The NF1 II/III binding site of JCV is also essential for both efficient transcription (the present study) and for replication (Sock et al, 1991). Interestingly, the integrity of the perfect palindromic NF1 II/III binding site was also found essential for the replication of JCV in vitro (Sock et al, 1991).

The proteins from HeLa and glial cells interact differently with JCV enhancer, thereby affecting the in vivo activity (Amemiya et al, 1989). The proteins interacting with

NF1 II site are different among different cell types. For example, two proteins of 230 and 85 kDa from HeLa and a protein of 45 kDa from glial cells specifically interact with the NF1 II/III motif of JCV (Khalili et al, 1988). Moreover, the 45 kDa brain cell-specific factor was purified and shown to support transcription of JCV in HeLa cells (Ahmed et al, 1990). Another cellular factor present in B-lymphocytes also interacts with JCV DNA (Major et al, 1990). However, no functional significance or studies on the expression of JCV enhancer in these cells are reported. One possibility is that, while factors from different cell types can physically interact with JCV control region, only brain-specific factors allow functional interaction. For muscle cell-specific gene regulation, a number of factors such as MyoD, myf-5 and the ubiquitously-expressed E2A gene products such as E12 and E47 bind the E-box. However, the muscle cell-specific factors but not the E2A products, are capable of activating transcription of muscle specific genes. Replacement of the basic region of MyoD with that of E12 results in failure of activation of transcription. This suggests that, although muscle-specific and E2A factors interact with the same DNA sequence, the functional consequences are different (Davis et al, 1990). Regulation of JCV expression might involve a similar mechanism.

An alternative possibility for JCV restricted cell

expression is that a glial cell-specific coactivator allows communication between NF1 and the basal transcription factors. The need for a coactivator has been suggested for several cell-specific genes (Weintraub et al, 1991; Mendel et al, 1991; Luo et al, 1992).

The archetype JCV predominantly recovered from kidneys has a Sp1 binding site located between TATA and NF1 II/III sequences. Also, NF1 II/III sequences are not duplicated (Yogo et al, 1990). This strain of JCV is transcriptionally inactive in the brain and is never detected there. The rearrangements in archetype sequences leads to the loss of the Sp1 factor binding site, generating a brain specific form capable of expression in glial cells (Ault and Stoner, 1993). This suggests that the interaction between factors binding to TATA box and NF1 II/III may be important for the glial cell-specific expression. For the archetype JCV, the presence of a Sp1 binding site between TATA and NF1 II/III may interfere with interaction between factors binding to NF1 sites, thereby rendering it incapable of expression in brain tissue. The interaction of a factor binding to NF1 II/III and TATA binding protein (TBP) may be facilitated by a coactivator. Such a coactivator may be present only in glial cells. Therefore, the binding activity detected in cell types other than glial cells would not be sufficient for expression of JCV. Further studies are needed to examine this possibility.

CHAPTER 4

Human JC virus nuclear factor 1 binding motif and large tumour (T-) antigen domains required for transactivation of late promoter.

4.1 Introduction

The regulatory region of JCV is contained mainly within the two 98 bp tandem repeats and is functional for both early and late genes. The early and late promoter-enhancer (JCV_E and JCV_L) control the expression of large and small T-antigens and viral structural proteins, respectively. Productive JCV infection of glial cells requires the JCV_L function that occurs subsequent to T-antigen synthesis.

Transregulation of JCV_L has been reported (Lashgari et al, 1989). As for JCV_E, the activity of JCV_L was also found to be restricted to glial cells. A low level of JCV_L activity was detected in the glial cells. In the presence of JCV T-antigen 9- to 12-fold more activity was observed in glial cells. Moreover, in nonglial cells JCV T-antigen activated the JCV_L to 4- to 8-fold. S1 nuclease mapping, and nuclear run on assays suggested that the effect of JCV T-antigen on late promoter was at the transcriptional level. Hence, it was concluded that the JCV T-antigen acts as a transactivator (Lashgari et al, 1989).

The HIV tat protein and JCV T-antigen activated the JCV_L. However, the effect of tat was several fold more than T-

antigen. T-antigen transactivated the late promoter significantly in both glial and nonglial cells. However, the tat protein of HIV activated JCV late promoter in glial cells while in nonglial cells very low levels of enhancement was noticed (Tada et al, 1990). Deletion analysis of JCV_L was used to localize the region(s) important for the transactivation of JCV_L by JCV T-antigen and HIV tat proteins. The results suggested that the region between -110 to -172 nt upstream of the start site is important for the transactivation of JCV_L and that the regions on JCV_L that are responsive to T-antigen and HIV tat protein are different. Hence, it was concluded that the JCV large T-antigen and HIV tat transactivate the JCV late promoter by different mechanisms (Chowdhury et al, 1990).

Another study used RA-differentiated P19 cells and reported transactivation and repression of JCV_L and JCV_E, respectively, by JCV T-antigen (Nakshatri et al, 1990a). However, the mechanism of transactivation of JCV_L by large T-antigen remained elusive. Specifically, the sequences of JCV_L and the amino acids of JCV T-antigen required for transactivation of late transcription remained unclear.

Another function of T-antigen is its transformation property. JC virus is oncogenic in various animals (Walker et al, 1973; Padgett et al, 1977; Varakis et al, 1978; London et al, 1978). Perinatal injection of JC virus into the brains of hamsters induced medulloblastomas (ZuRhein and Varakis, 1979;

ZuRhein, 1983). In humans, post mortem analysis of CNS tissues of patients with PML revealed the presence of several glial tumours (Sima et al, 1983). However, evidence that these tumours were caused by JCV was not clear.

The Tokyo-1 strain of JC virus isolated from a PML patient in Japan exhibited high neuro-oncogenicity in hamsters and 95% of the injected animals developed medulloblastomas (Nagashima et al, 1984). The medulloblastomas developed in hamsters resembled human medulloblastomas histologically, immunohistochemically and ultrastructurally. Tumours in both species demonstrated highly malignant characteristics. However, while all the tumours induced in animals expressed T-antigen of JCV, this antigen is not detected in human brain tumours. Importantly, JC virus is the only human virus known to be able to cause solid tumours in non-human primates (London et al, 1978; Reith et al, 1980).

Although JCV readily induces tumours in experimental animals, it transforms cells in culture in vitro inefficiently (Frisque and White, 1992). Transformation efficiency of JCV T-antigen is poor as compared to those of other DNA tumour viruses such as, SV40 and BKV (Nakshatri et al, 1988; Haggerty et al, 1989; Fanning, 1992). Early studies indicated that JCV transforms primary human fetal glial cells (PHFG) and human vascular endothelial cells (Fareed et al, 1978; Walker and Padgett, 1978). Later it was shown that PHFG cells were

transformed by origin-defective mutants of JCV to create the POJ cell line (Mandl and Frisque, 1986). Primary hamster brain cells were transformed by infection with Mad1, Mad2, Mad3, and Mad4 viruses and by transfection with Mad1 DNA. The transformants exhibited several features characteristic of the transformed phenotype which include high saturation densities, increased growth rates in high and low serum concentrations and anchorage-independent growth. Baby hamster kidney cells (BHK-21) and rat fibroblasts (Rat2) were transformed in culture (Bollag et al, 1989; Haggerty et al, 1989).

The JCV genome was detected in transformed cells as multiple copies in a tandem head-to-tail arrangement (Mandl and Frisque, 1986; Mandl et al, 1987). Southern blot hybridization indicated that JCV DNA integrates randomly into the host chromosomes and free, unintegrated viral genomes have not been observed (O'Neill et al, 1988). However, the large T-antigen sequences important for transformation of cells remained unclear.

The objectives of my study are as follows.

1) To examine the role of NF1 binding motifs in the transactivation of JCV_L by T-antigen.

2) To investigate the ability of these motifs to interact with factors in the presence and absence of T-antigen.

3) To delineate the amino acid sequences of large-T antigen required for transformation and transactivation of JCV_L.

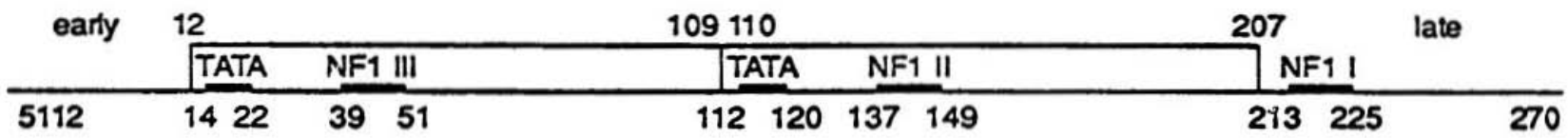
4.2 Results

4.2.1 NF1 motifs required for JCV_L transactivation.

To examine the role of NF1 binding sites in the transactivation of JCV_L by large T-antigen, site-directed mutagenesis was performed (Fig. 4.1). Reporter plasmids containing the various mutations were transfected into RA P19 cells with or without T-antigen expression plasmid. A substantial transactivation of pJC_L cat (WT) was obtained by cotransfecting with T-antigen expression plasmid (Fig. 4.2 WT). The mutated NF1 I site (I) construct also showed substantial transactivation by T-antigen (Fig.4.2, I). Next, the role of NF1 II/III was examined. Mutations in NF1 II greatly reduced transactivation in RA P19 cells. Mutations in NF1 II plus III and NF1 I, II, plus III eliminated transactivation (Fig. 4.2, II.III and I.II.III, respectively). The results indicated the necessity of the NF1 II/III binding motifs located in the 98 bp repeats, but not NF1 I, for transactivation of JCV_L by T-antigen in RA P19 cells. The effect of NF1 mutations on the transactivation of JCV_L by T-antigen was also tested in the DMSO-differentiated P19 muscle cells. The WT promoter and NF1 I mutant (I) were repressed by T-antigen whereas, the NF1 mutants II, II.III and I.II.III were not affected in the presence of T-antigen (Fig. 4.3). The results suggested that the JCV T-antigen activates JCV_L in RA P19 cells and represses JCV_L in nonglial cells.

Fig. 4.1 Wild type and mutated NF1 motifs in JCV_L sequences in CAT plasmids.

Top: Diagram of JCV_L sequences in the JCV fragments inserted into pSV0cat. The early HindIII and late PvuII sides are indicated. TATA boxes and NF1 site motifs are indicated and delineated by thick lines. The nt positions are indicated. The boxes represent the 98 bp tandem repeats. Bottom: List of mutated plasmids and mutated sequences for the three NF1 sites. Mutated NF1 nt are indicated by lower case letters. Wild type NF1 nt are indicated in pJC_L cat by upper case letters and in other plasmids by dashes. Inverted palindromic sequences are underlined for pJC_Lcat (WT).



Plasmids	NF1 III	NF1 II	NF1 I
pJC$_L$cat (WT)	TGGCTGCCAGCCA	TGGCTGCCAGCCA	TGGAAAGCAGCCA
pJC$_L$cat I	----------	----------	-a--------a-c
pJC$_L$cat II	----------	gta-------a-c	----------
pJC$_L$cat II.III	gta-------a-c	gta-------a-c	----------
pJC$_L$cat I.II.III	gta-------a-c	gta-------a-c	-a--------a-c

Fig. 4.2 Effect of NF1 mutations on JCV_L transactivation by T-antigen in RA-treated P19 cells.

Plasmids were wild type $pJCV_L$cat (WT) and others with NF1 site mutations, as indicated by the unique letters for the constructs described in Fig. 4.1. Transactivation is the difference between CAT activity (% conversion) in the absence and presence of JCV T-antigen expression plasmid. CAT activity was normalized with β-galactosidase. Results are averages of three experiments. Percent CAT conversion in T-antigen nonexpressing cells: WT, 5.9; I, 6.6; II, 6.2; II.III, 4.2, I.II.III, 8.4.

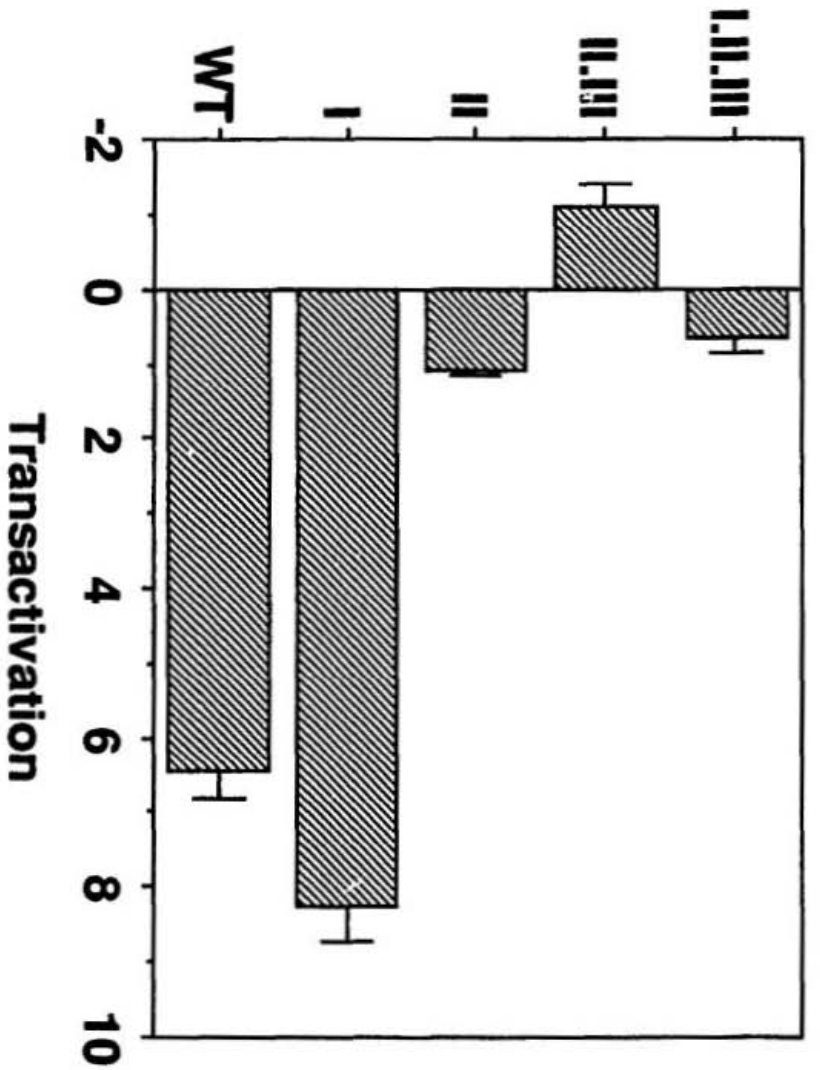
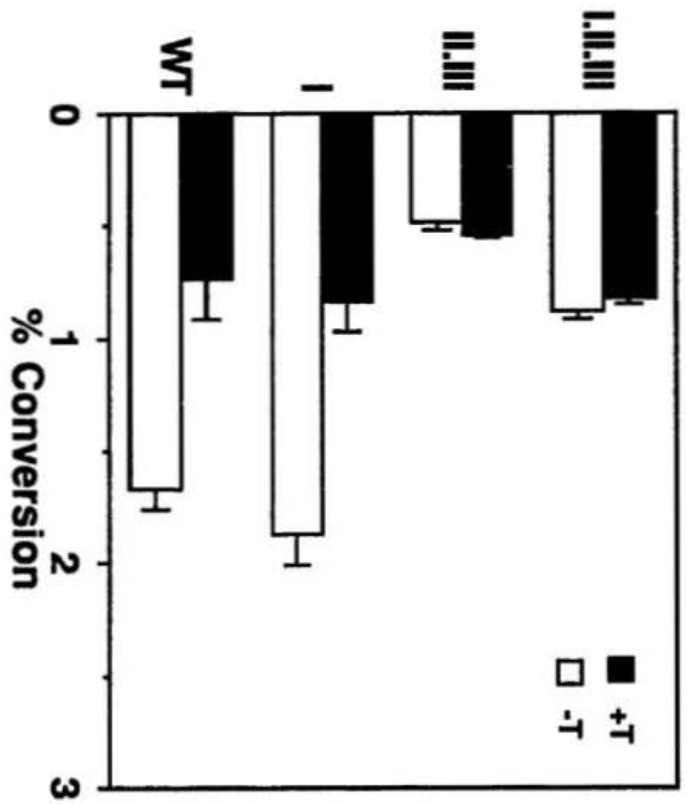

WT
I
II
II,III
I,II,III
-2
0
2
4
6
8
10
Transactivation

Fig.4.3 Effect of NF1 mutations on JCV_L transregulation by T-antigen in P19 muscle cells.

Plasmids are as explained for Fig. 4.2. Other methods are the same as in Fig. 4.2. In the figure % conversion is shown instead of transactivation described for Fig. 4.2 as repression of late promoter in the presence of T-antigen was noticed in contrast to activation in P19 RA cells.


WT
I
II,III
I,II,III
0
1
2
3
% Conversion
-T
+T

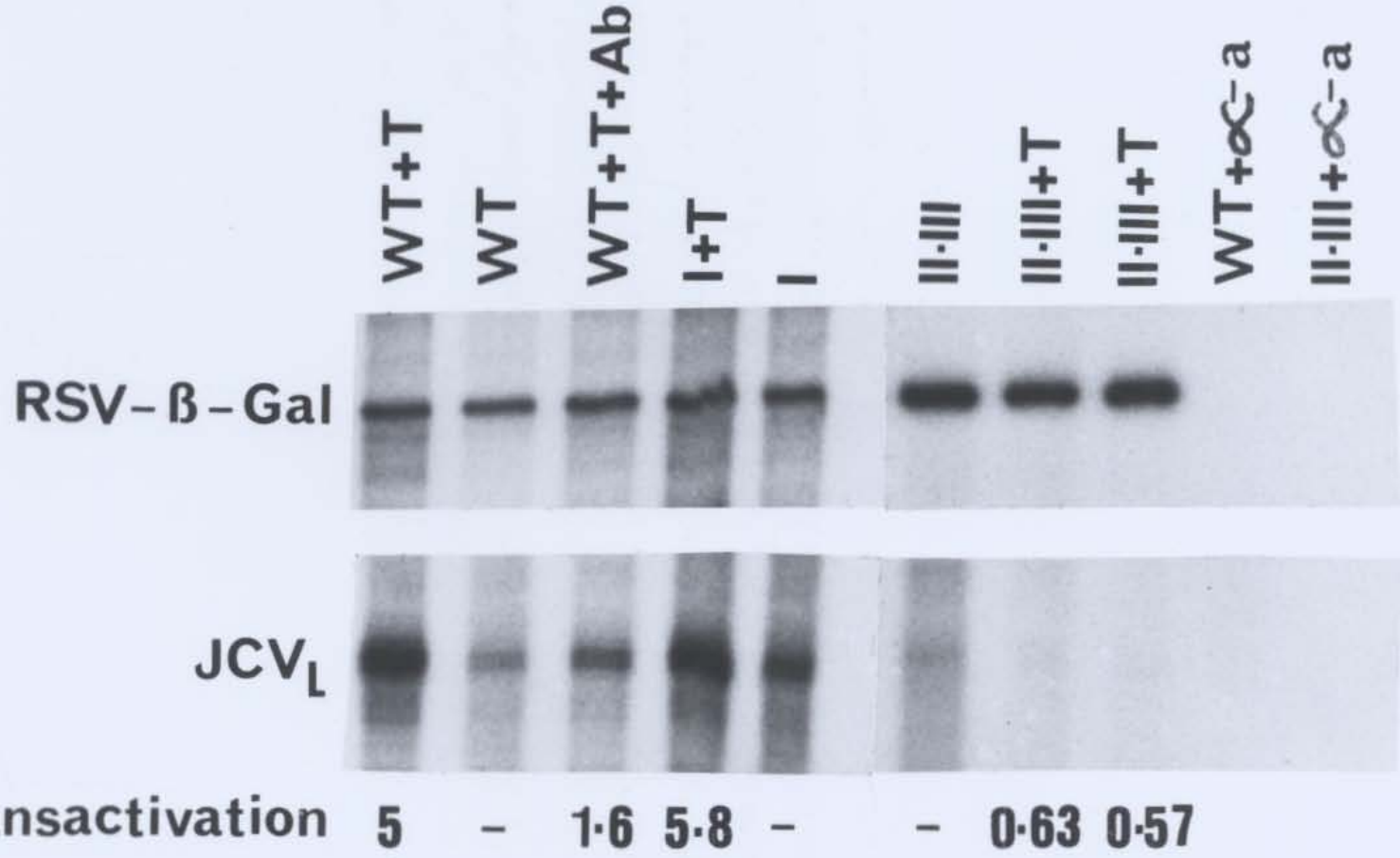
The role of NF1 sites in late promoter function was also examined by *in vitro* transcription assays. In the presence of P19 RA cell extract from cells which expressed JCV T-antigen, JCV_L wild type (WT) and mutated NF1 I promoters were transcribed at 5-fold and 5.8-fold greater rates, respectively, compared with extract from non-T-antigen expressing glial cells. Monoclonal antibodies against large T-antigen reduced JCV_L transactivation to 1.6-fold, confirming the involvement of T-antigen and indicating that T-antigen acted *in vitro*. Mutations in the NF1 II plus III sites resulted in 0.6-fold transactivation, a 1.7-fold repression of $pJCV_L$ (Fig. 4.4). The transcription was by RNA polymerase II, because alpha-amanitin blocked all transcriptional activities. Thus, the *in vitro* transcription assays confirmed the requirement of NF1 II and III, but not I, for transactivation of JCV_L by large T-antigen in P19 RA cells.

4.2.2 Mediation of JCV large T-antigen effect through NF1 II/III

Mobility shift assays were used to examine whether the induction of JCV_L by T-antigen involved alterations in the binding of the NF1 sites of the 98 bp repeats. For normal P19 RA cell extract, the 98 bp repeat probe with wild type NF1 II/III was bound in an upper complex and a doublet of lower complexes. Extracts of T-antigen expressing P19 RA cells gave a binding pattern in which the upper complex was increased

Fig. 4.4 Effect of T-antigen and JCV NF1 sites on JCV_L <u>in vitro</u> transcription.

Names of the plasmids are as described in Fig. 4.1 and 4.2. Glial cell extracts had 75 μg protein. Labels: T, cells transfected with T-antigen expression plasmid; Ab, crossreacting SV40 monoclonal T-antigen antibody in assays; α-a, 1 μg/ml alpha amanitin polymerase II inhibitor; RSV-β-Gal, β-galactosidase internal control RNA; JCV_L, JCV_L RNA; Transactivation, fold transactivation by T-antigen. Pairs of assays with extracts for cells expressing T-antigen and not expressing T-antigen were compared for fold transactivation values. Results were consistent for two experiments, including greater transactivation for I.


WT+T
WT
WT+T+Ab
I+T
I
II·III
II·III+T
II·III+T
WT+∝-a
II·III+∝-a
RSV-ß-Gal
JCV$_L$
Transactivation
5
–
1·6
5·8
–
–
0·63
0·57

7.2-fold (Fig. 4.5, T). The increased binding for the upper complex was due to the immediate function of the T-antigen, because monoclonal antibodies for T-antigen blocked the increase (Fig. 4.5, T+Ab(S)). Non-specific monoclonal antibodies against MHC class II ($I\text{-}A^K$) gene product failed to block the increased binding of the upper complex (Fig. 4.5, T+Ab(NS)).

To further examine the effect of T-antigen on the binding activity, mobility shift assays were done with wild type and mutated NF1 II/III oligonucleotide probes. Only one complex was observed with WT NF1 II/III olgonucleotide probe and T-antigen increased the amount of this complex by 6.2-fold, similar to the increase for the upper complex with 98 bp repeat probe (Fig. 4.6, WT, lane T). Monoclonal antibodies against T-antigen blocked the increase in the complex (Fig. 4.6, WT, lane T+Ab(S)). The monoclonal antibody against MHC class II gene product had no effect (Fig. 4.6, WT, lane T+Ab(NS)). Mutated NF1 II/III oligonucleotide was not bound, confirming the specificity of binding to this motif (Fig. 4.6, mu). These results confirmed that T-antigen facilitated the binding and that the NF1 II/III motif is involved in binding. Overall, the results suggested that transactivation increased the formation of a productive transcription complex at JCV_L due to an interaction(s) with the NF1 II/III motif.

Fig. 4.5. Effect of T-antigen on binding of cellular factors to JCV 98 bp repeat regulatory region.

Mobility shift assays used the StyI 98 bp repeat fragment of JCV regulatory region and 8 μg of protein. Labels: F, free probe with no protein; B, binding for non-T-antigen expressing glial cell extract; T, T-antigen expression plasmid-transfected cell extract; T+Ab (S), T+ monoclonal T-antigen antibody in assays; T+Ab (NS), T+ nonspecific control MHC monoclonal antibody. Fold change in binding of upper complex in the presence of T antigen, in comparison with lane B which used extract with no T-antigen, are T, 7.2-fold; T+Ab (NS), 6.4-fold; and T+Ab (S), 0.9-fold, as determined by densitometry.

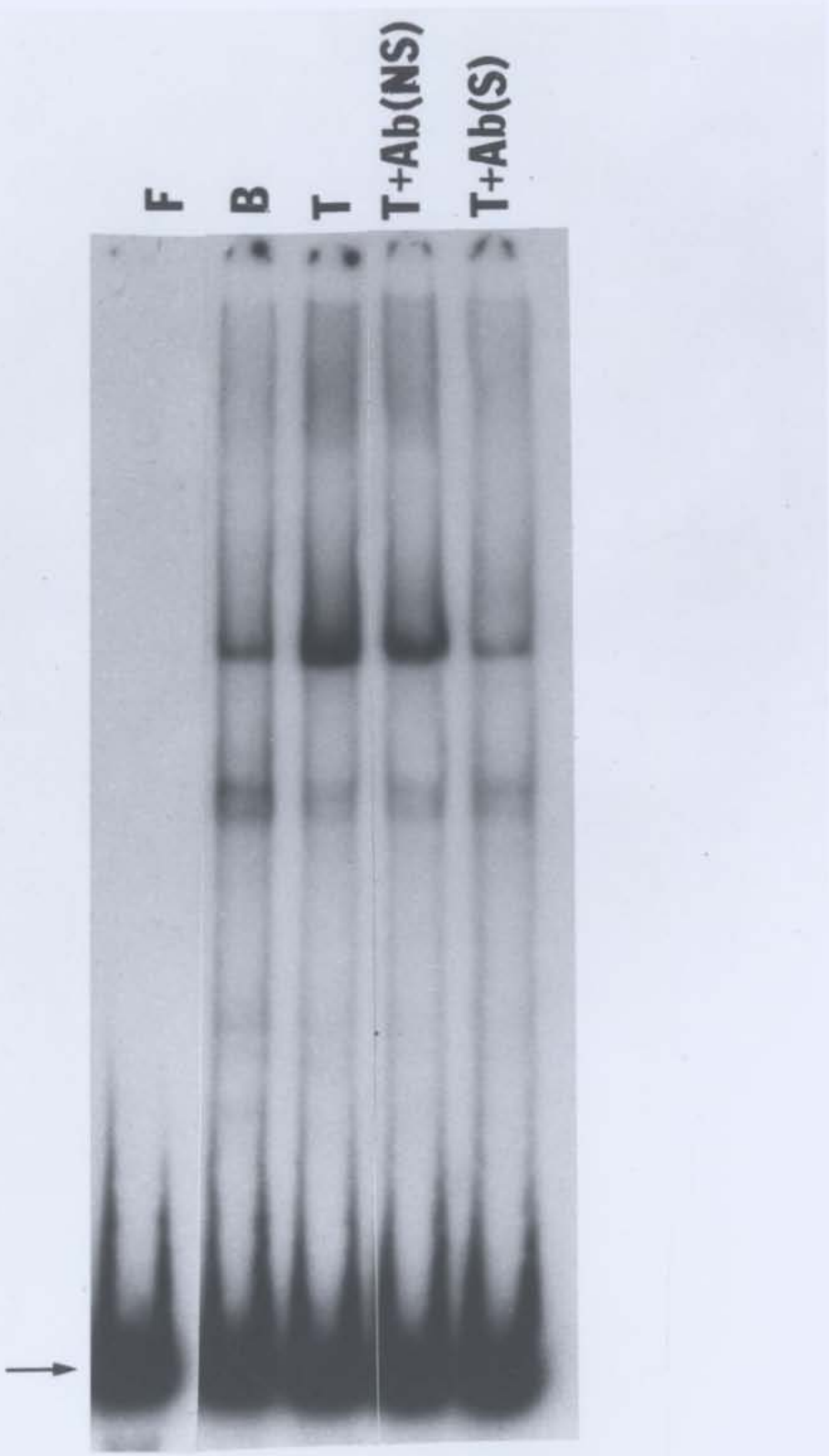
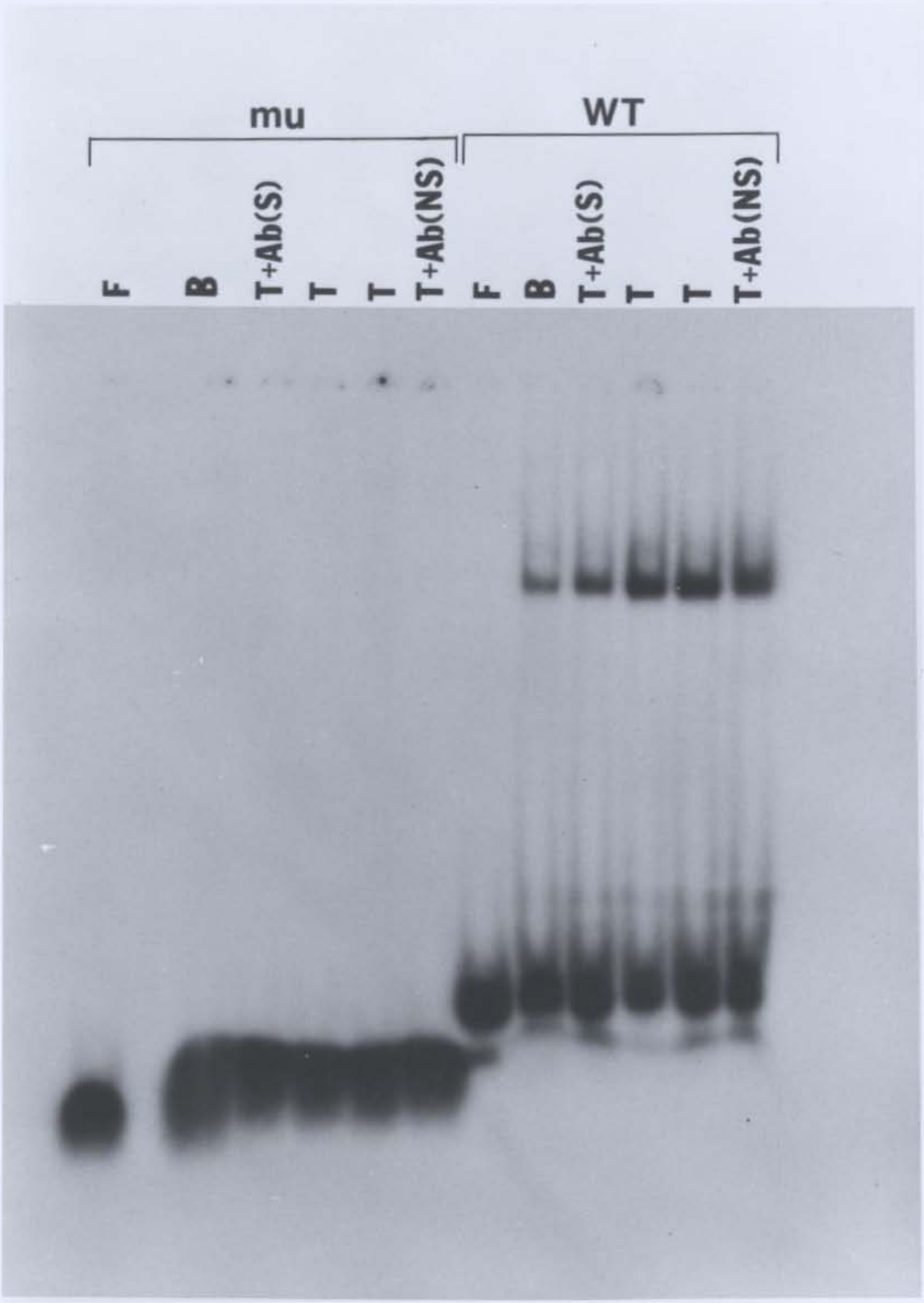

F
B
T
T+Ab(NS)
T+Ab(S)

Fig. 4.6. Effect of T-antigen on NF1 binding.

Mobility shift assays used wild type (WT) and mutated (mu) NF1 II/III oligonucleotide probes and 6 μg of protein.

Labels: F, free probe with no protein; B, binding for non-T-antigen expressing glial cell extract; T, T-antigen expression plasmid-transfected cell extract; T+Ab (S), T+ monoclonal antibody against T-antigen; T+Ab (NS), T+ nonspecific control MHC monoclonal antibody. Densitometrically determined fold increases relative to lane B for nonexpressing cell extract and WT probe: T+Ab (S), 2.1; T, 6.2; T, 6.4; and T+Ab (NS), 5.6.


mu
WT
F
B
T+Ab(S)
T
T
T+Ab(NS)
F
B
T+Ab(S)
T
T
T+Ab(NS)

4.2.3 T-antigen domains for JCV_L transactivation and transformation

The amino acid domain(s) of JCV T-antigen necessary for transactivating JCV_L were also examined. Ten translation termination linker and deletion mutations in the T-antigen expression plasmid were generated (Fig. 4.7). See Fig. 1.1 for a comparison of T-antigen with the wnole JCV genome. Wild type and mutated expression plasmids were co-transfected with the wild type reporter plasmid. Mutated constructs encoding the first 10, 141, 180, and 195 amino acids (pK4984, pK4246, pK4129, and pK4085, respectively, Fig. 4.7) transactivated poorly (Fig. 4.8). Constructs encoding the first 437, 552, and 591 residues (pK3359, pK3013, and pK2896, respectively, Fig. 4.7) showed nearly wild type activation levels (Fig. 4.8). The internal deletions for amino acids 142-180 (pKΔ117) and 196-437 (pKΔ726) reduced transactivation to a half and a third, respectively, of wild type (Fig. 4.8). Taken together, the results suggested that amino acid residues 196-437 are necessary for most of the wild type transactivating activity.

Transformation is an important function of T-antigen and protein domains in general are often functional for different or related biological functions. Therefore, primary BRK cells were assayed for transformation with the same T-antigen domain mutants used for the transactivation studies. The results of the transformation assays indicated that the

Fig. 4.7. Diagram of mutated T-antigen sequences.

Expression plasmids are shown. Numbers in plasmid names correspond to sites of linker insertion and number of nt deleted. The pKΔ1945 deletion is out-of-frame. Labels: numbers in diagram, JCV nt positions; ATG, initiation codon; TAA, termination codon; TAG and arrow heads, translation termination linker insertion sites; solid lines, JCV T-antigen ORF; dashed lines, intron sequences; black boxes, deleted sequences.

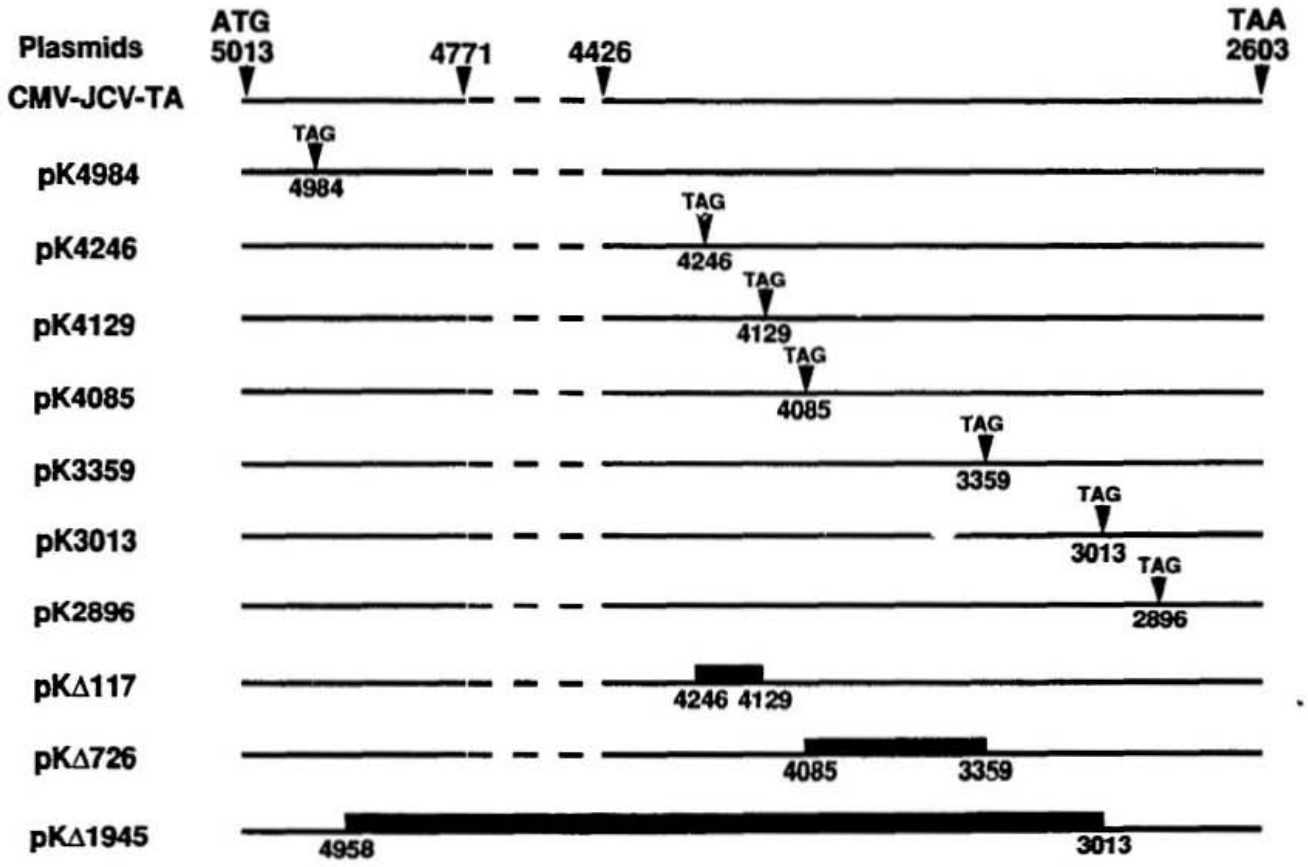

Plasmids
ATG
5013
4771
4426
TAA
2603
CMV-JCV-TA
TAG
pK4984
4984
TAG
pK4246
4246
TAG
pK4129
4129
TAG
pK4085
4085
TAG
pK3359
3359
TAG
pK3013
3013
TAG
pK2896
2896
pKΔ117
4246 4129
pKΔ726
4085
3359
pKΔ1945
4958
3013

Fig. 4.8. Transactivation of JCV_L by mutated T antigen expression plasmids.

WT indicates wild type CMV-JCV-TA. Other labels and estimate of transactivation are as in Fig. 4.2 and 4.7. CAT expression values in T-antigen nonexpressing cells: WT, 5.1; pK4984, 4.7; pK4246, 5.2, pK4129, 5.7; pK4085, 5.0; pK3359, 5.0; pK3013, 4.8; pK2896, 5.0; pKΔ117, 5.05; pKΔ726, 5.55; pKΔ1945, 4.9.

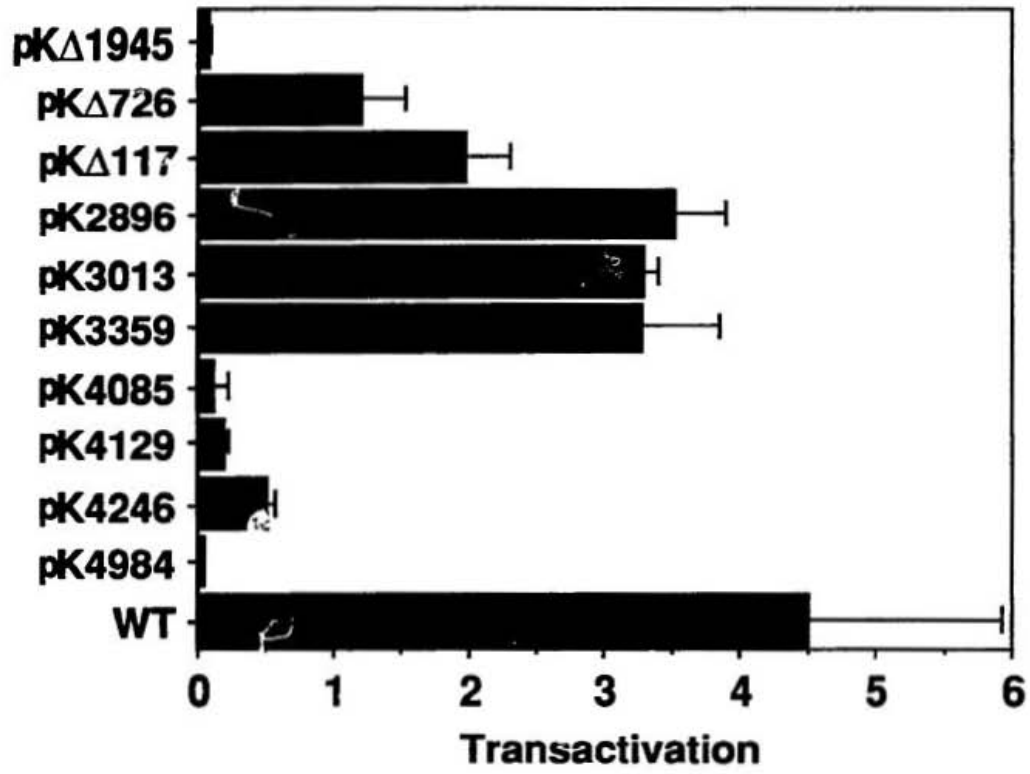

pKΔ1945
pKΔ726
pKΔ117
pK2896
pK3013
pK3359
pK4085
pK4129
pK4246
pK4984
WT
0
1
2
3
4
5
6
Transactivation

Table 4.1 Transformation domains of JCV T antigen.

Plasmids[a]	Transformation of BRK cells[b]				Number of amino-acids in T antigen[c]
	Exp.1	Exp.2	Exp.3	Mean	
CMV- JCV- TA	17	28	21	22	1 - 688
pK4984	0	0	0	0	1 - 10
pK4246	10	6	8	8	1 - 141
pK4129	3	5	4	4	1 - 180
pK4085	2	4	3	3	1 - 195
pK3359	16	19	10	15	1 - 437
pK3013	16	21	11	16	1 - 552
pK2896	14	27	14	18	1 - 591
pKΔ117	9	16	12	12	1 - 141; 181 - 688
pKΔ726	2	5	2	3	1 - 195; 438 - 688
pKΔ1945	0	0	0	0	1 - 19; (552 - 688)

[a] The indicated plasmids are as shown in Fig. 4.7

[b] BRK cells were plated in three 50 mm plates. After 24h, the cells were co- transfected with 5 μg/plate of the indicated plasmids and the same amount of EJ-Ha- *ras*-1-pML.

[c] The bracketed amino acids of pKΔ1945 are out-of-frame.

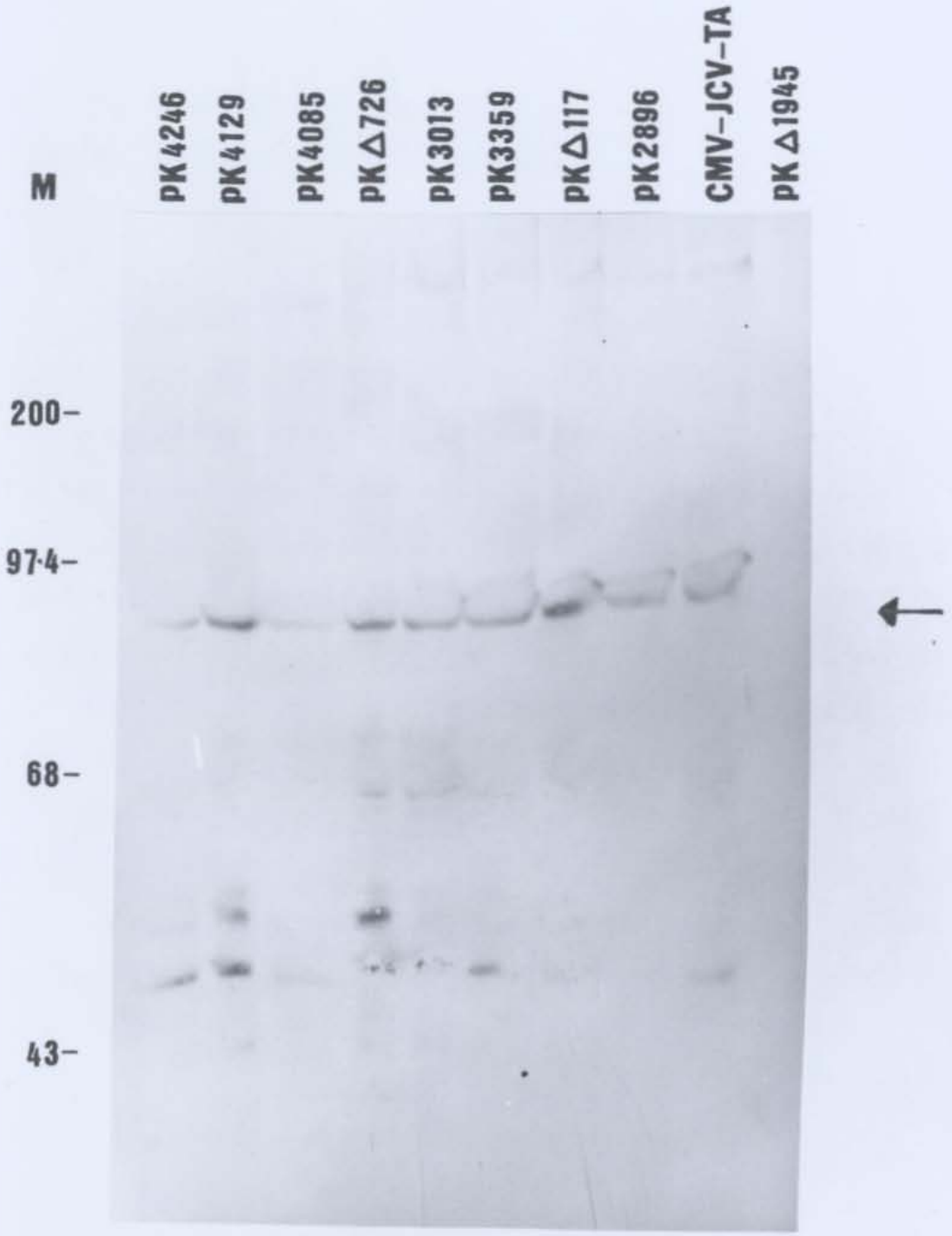
transformation domain was mostly in amino acid residues 196-437 (Table 4.1). The requirement of domains was similar to that for transactivation, considering the distinct nature of the two assay systems.

4.2.4 Western blot analysis of WT and mutant T-antigen expression plasmids.

The difference in transactivation observed between wild type and mutated T-antigen expression plasmids could have been due to instability of the mutated proteins. Therefore, lysates from P19 glial cells transfected with these plasmids for expressing T-antigen were examined in Western blot assays for T-antigen protein. For only the pKΔ1945 out-of-frame deletion mutation encoding the first 19 amino acids (Fig. 4.7) no JCV T-antigen protein was observed (Fig. 4.9). T-antigen was stably produced at comparable rates from the other 9 plasmids, since the protein was present at comparable levels in the glial cells (Fig. 4.9). The size of T-antigen for the various mutated constructs was similar to WT. This is most probably due to the posttranscriptional modifications of T-antigen *in vivo*, as also reported for BKV T-antigen (Nakshatri *et al*, 1988). Taken together, the results indicate that the effect of T-antigen mutations on transactivation and transformation are due to functional T-antigen protein domains (Fig. 4.8; Table 4.1).

Fig. 4.9. Western blot analysis of T-antigen proteins expressed from mutated plasmids.

JCV T-antigen is detected as the band corresponding to the arrow.The labelling is the same as described for Fig. 4.7. Molecular weight markers (M) are indicated in kDa. Lower bands may be degradation products of T-antigen.


M
PK4246
PK4129
PK4085
PKΔ726
PK3013
PK3359
PKΔ117
PK2896
CMV-JCV-TA
PKΔ1945
200-
97·4-
68-
43-

4.3 Discussion

The studies of this chapter examined the molecular basis for induction of late JCV gene expression in RA P19 cells by the transactivating early gene product, large T-antigen. The involvement of the NF1 motifs of the JCV late region was approached by site-directed mutagenesis of NF1 motifs. The results clearly showed that the integrity of the two NF1 sites within the 98 bp repeat, but not the third site located towards the late side, was essential for the transactivation of JCV late promoter-enhancer in glial cells (Figs 4.2 and 4.4). Transactivation of JCV_L by T-antigen required direct T-antigen function during the assay, since antibody to T-antigen abolished transactivation in vitro (Fig. 4.4). This indicated that the mechanism did not involve alterations such as the level of *in vivo* synthesis of NF1 or other transcription factors. The *in vitro* gel shift assays clearly suggested that T-antigen mediates this effect by facilitating the increased binding of NF1. Further, *in vitro* binding assays with oligonucleotide probes confirmed the requirement of the specific NF1 II/III motifs.

Transactivation of JCV late promoter by T-antigen in the glial cells was clearly shown with 5-fold higher activity in the presence of JCV T-antigen in *in vitro* transcription assays. The NF1 II and III were distinctly important. These results are in agreement with the previous observations that

JCV T-antigen mediates its transactivating effect through the region between nt -110 to -172 (Chowdhury et al, 1990). The results from the present study clearly narrowed down this region to NF1 II/III binding sites (nt -137 to -149 and -39 to -51, respectively). The mutation of only NF1 site II eliminated most of the transactivation. Therefore, the duplicated NF1 of the 98 bp repeat appears to be important for expression in glial cells. NF1 II/III is included in the 62 bp region that was previously shown to be required for glial cell-specific transactivation (Chowdhury et al, 1990). Moreover, the JCV genotypes identified in brain tissue consistently harboured duplications of the sequences that specifically include 12-40 nt containing this NF1 site. In addition, in the archetype JCV which is frequently present in the kidney, the corresponding NF1 site is never duplicated (Yogo et al, 1990). This suggests there are kidney-specific NF1 functions. NF1 is a family of proteins, and distinct factors have been shown previously to regulate gene-specific expression in other tissues such as liver (Jackson et al, 1993). Although the NF1 motifs in the 98 bp repeats are shown here to be essential for the transactivation of JCV late promoter, my results do not rule out the requirement for other sequences in the late promoter.

Earlier, several studies reported different start sites for JCV late transcription and the results were ambiguous

(Kenney et al, 1986b; Khalili et al, 1987). The results of in vitro transcription assays of the present study provided precise and important information on the location of the start site of JCV late transcription. Based on the size of the transcript generated, the start site for late transcription is centred around nt 200-203, as suggested by primer extension and S1 nuclease in the studies of Daniel and Frisque (1993).

The NF1 binding motifs (NF1 II/III) in the JCV regulatory region are important for both early and late gene expression of JCV (Kerr and Khalili, 1991; results of the chapter and chapter 3). To examine the molecular mechanism, an attempt was made to look at alterations in the binding activity of NF1 in the presence of T-antigen. The results of mobility shift assays demonstrated that T-antigen increased the binding of NF1 to the NF1 motifs located in the repeats.

JCV is oncogenic in animals and it is the only human virus known to cause solid tumours in non-human primates (London et al, 1978; Reith et al, 1980). Hence the JCV T-antigen amino acid sequences important for transformation were investigated by constructing and studying a panel of T antigen mutants. The present study showed that the transactivation and transformation domains of JCV large T-antigen are similar, considering the distinct nature of the two assay systems (Fig. 4.8; Table 4.1).

Parental and chimeric DNAs of JCV, BKV, and SV40 have been used to show that both the regulatory sequences and the large T-antigen amino terminus sequences are necessary for the JCV restricted transformation behaviour. Characterization of the cloned cell lines prepared from T-antigen transformants revealed variations in the amount and stability of T-antigen. Ability of T-antigen to form stable complexes with p53 was also affected (Bollag et al, 1989; Haggerty et al, 1989). The tumour suppressor, p53, is important for the control of cell proliferation (Milner, 1991; Levine 1992). JCV T-antigen was also shown to interact with Rb and p107 (Dyson et al, 1989, 1990). Rb and p107 are involved in control of cell cycle progression and Rb blocks the cell cycle at a certain point in G1. JCV T-antigen interacts with all these proteins and possibly perturbs the action of these proteins, thereby resulting in the loss of control on cell proliferation. The mutants pKΔ117 and pKΔ726 used in my study showed reduction in their transformation potential (Fig. 4.7; Table 4.1). The mechanism of this reduction in transformation most probably involves loss of T-antigen domain(s) important for binding to one of these proteins involved in the control of cell proliferation.

The differences in transactivation and transformation between WT and mutant JCV T-antigens could have been due to variations in the stability and the levels of mutant T-

antigen. However, results of Western blot analysis revealed no change in either stability or levels of T-antigen protein (Fig. 4.9). The mutant T-antigen expression plasmid expressing the first 141 amino acids (pK4246) showed more transformation efficiency than those expressing the first 180 (pK4129) and 195 (pK4085) amino acids (Table 4.1). It is possible that the T-antigen sequences located between nt 4246 and 4085 contain an inhibitory domain. The interplay between activation domain located within nt 4085-3359 for amino acids 196-437 and the inhibitory domain within nt 4246-4085 may determine the transformation potential of JCV T-antigen. This can be supported by the observation that adenovirus E1a, an oncogenic protein, contains sequences in the carboxyl (C-) terminus responsible for inhibiting its transformation potential (Subramanian et al, 1989). The suppressive effect of the C-terminal region of E1a protein on ras-mediated transformation was due to interference with the ras-directed transformation pathway or abrogation of E1a cooperation with ras (Subramanian et al, 1989). Hence, the possible interactions between different regions of JCV T antigen, the cellular proteins such as Rb, p107, p53 and ras oncogene lead to oncogenic transformation in a complex process.

A similar cell system to that of my studies was used to study the BK virus large T-antigen domain important for transformation. The BKV T-antigen domain was mapped to amino

acids 265-417 (Nakshatri et al, 1988). My studies show that JCV T-antigen amino acids 196-437 are important for transformation (Fig. 4.7, Table 4.1). Therefore, the transformation domain of T-antigens of JCV and BKV appear to be within the same regions. This is not surprising since the T-antigens of these viruses share a high percentage (85%) of homology. The transformation domains of T-antigens of SV40 and BK viruses are different and share only 54% homology (Nakshatri et al, 1988).

The domain of T-antigen required for transactivation was mapped with a panel of T-antigen mutant constructs. Most of the function was localized to the middle third of T-antigen and the amino terminus (Fig 4.7 and 4.8). The overall homology among the genomes of JCV, BKV and SV40 is high (Frisque et al, 1984). However, homology in the regulatory region is below average and only JCV and BKV have NF1 sites. The relative expression level of JCV was 24-fold more than that for SV40 for glial cells compared with muscle cells, and 6-fold more than that for BKV (Nakshatri et al, 1990, 1991). The difference from BKV could be due to the unique productive interaction of the different JCV NF1 sites with a distinct NF1. Alternatively, the greater number of motifs, such as the GC-rich sequences in BKV, might alter or conceal a similar NF1 specificity to glial cells. Consistent with the latter possibility, interactions with BKV NF1 sites were specifically

weaker in muscle cell extracts than in glial cell extracts (Nakshatri et al, 1991).

The TATA-less SV40 late promoter functions efficiently through a coactivator interacting with Sp1 which recognizes the SV40 GC-rich motif (Pugh and Tjian, 1990). The BK GC-rich sequences may also function through a similar mechanism. However, JCV has no GC-rich sequences and has much lower activity than SV40 and BKV, even in P19 glial cells (Nakshatri et al, 1990a). Therefore, the brain-specific expression of JCV appears to be relatively more dependent on the NF1 II/III sites.

In contrast to JCV, SV40 has no glial cell specificity. Transactivation of the late promoter of SV40 has been attributed to TEF-1 (Coulombe et al, 1992b; Gruda et al, 1993). SV40 T-antigen was suggested to transactivate the SV40 late promoter as a coactivator through its interaction with TEF-1 and TATA binding protein, TBP (for detailed explanation refer to subsection 1.7.2.2.1.1 and 1.7.2.2.1.2). Although SV40 differs from JCV by having TEF-1 sequences and no NF1 site, the greater information available for SV40 warrants a comparison with JCV. JCV and SV40 T-antigens have 72% homology, compared to 83% between JCV and BKV (Frisque et al, 1984). The domain of SV40 T-antigen that binds TBP is within amino acids 5-172, a region with approximately 40% of wild type transactivation activity (Gruda et al, 1993); the JCV

amino acids 1-141 had 12% of wild type transactivation (Fig. 4.7 and 4.8). The domain of SV40 T-antigen that binds TEF-1 and TBP is within amino acids 5-383, and has the most of wild type transactivation. Similarly, the JCV amino acids 1-437 had equal activity to wild type and the deletion of amino acids 196-437 disrupted most of the transactivation.

The JCV amino acids 196-437 region is of further interest, because it also corresponds to the SV40 TEF-1 binding domain and the nt 302-320 zinc finger domain (Fanning, 1992). Whereas the DNA binding domain from nt 131-259 of SV40 probably binds TBP and has an average homology to JCV, the zinc finger domain has a marginal 39% homology. The homology between JCV and BKV is 78% for this region, possibly reflecting a similar mechanism for both of these human viruses. This is still significantly different from the 91% homology for the DNA binding domains between JCV and BKV. There is no quantitative data to suggest that T-antigen was stably produced at comparable rates from the T-antigen mutants. Hence a cell line that constitutively produce JCV T-antigen and the cells transformed by mutant T-antigen expressing plasmids generated in the present study can be used to solve this problem.

The exact mechanism by which T-antigen activates the JCV_L is not clear. One possibility is that NF1 protein may be modified by JCV T-antigen leading to transactivation of JCV_L,

even though no obvious differences in the mobility of complex in the presence of T-antigen were detected (Fig. 4.5 and 4.6). However, the possibility of post-translational modification of NF1 by T-antigen can not be ruled out.

Another possibility is that JCV T-antigen might be acting as an intermediary molecule between NF1 and one of the basal transcription factors for late promoter activation, analogous to adenovirus E1a (Scholer et al, 1990; Lee et al, 1991) and SV40 T-antigen (Gruda et al, 1993). However, mobility shift assays using the 98 bp repeat failed to reveal any obvious difference in the mobility of the complexes in the form of a supershift. However, the ternary complex containing T-antigen could be unstable and dissociate during the native gel electrophoresis. There are several examples for this. HTLV tax protein increases the rate of transcription by facilitating the binding of the factor ATF. Although the tax protein interacts with ATF in vitro, no change in the mobility of the complex containing ATF was observed, either in the presence of tax or with antibodies against tax protein (Franklin et al, 1993; Wagner and Green, 1993). The authors suggested that with the gel conditions used, the complexes might have dissociated. With respect to the glucocorticoid receptor (GR), although it interacts with purified AP-1 in vitro, no further retardation of the fos/jun complex was observed in the presence of GR in mobility shift assays. The

lack of supershift was suggested to be due to disruption of GR during gel resolution (Konig et al, 1992). Instability of ternary complexes in gel shift assays has been reported in other cases such as interaction between oct-1 and GR (Kutoh et al, 1992), SRF Serum response factor and Phox1 homeodomain protein (Grueneberg et al, 1992) and NF-IL6 Nuclear Factor for interleukin-6 expression and GR (Nishio et al, 1993). Hence, the possibility that T-antigen interacts directly with NF1 and the basal transcription factors can not be excluded, simply based on the failure to detect supershifting in gel shift assays (Fig. 4.5).

The JCV late promoter is down-regulated by LCP-1 56 kDa repressor protein (see the following paragraph) which interacts with a pentanucleotide repeat sequence (AGGGAAGGGA which is indicated as NRE in Fig. 1.2). This sequence is located adjacent to NF1 binding sites in the repeats (Tada et al, 1991; Tada and Khalili, 1992). Another possible mechanism for transactivation of late promoter by T-antigen might involve changes in the balance of the interaction of this repressor protein with NF1 II/III and pentanucleotide repeat sequence, leading to the increased binding to NF1 II/III observed in gel shift assays (Fig 4.6). This might involve a mechanism in which NF1 and repressor compete for binding JCV NF1 site and NRE, respectively. Therefore, the effect of T-antigen might be to relieve this competition by preventing the

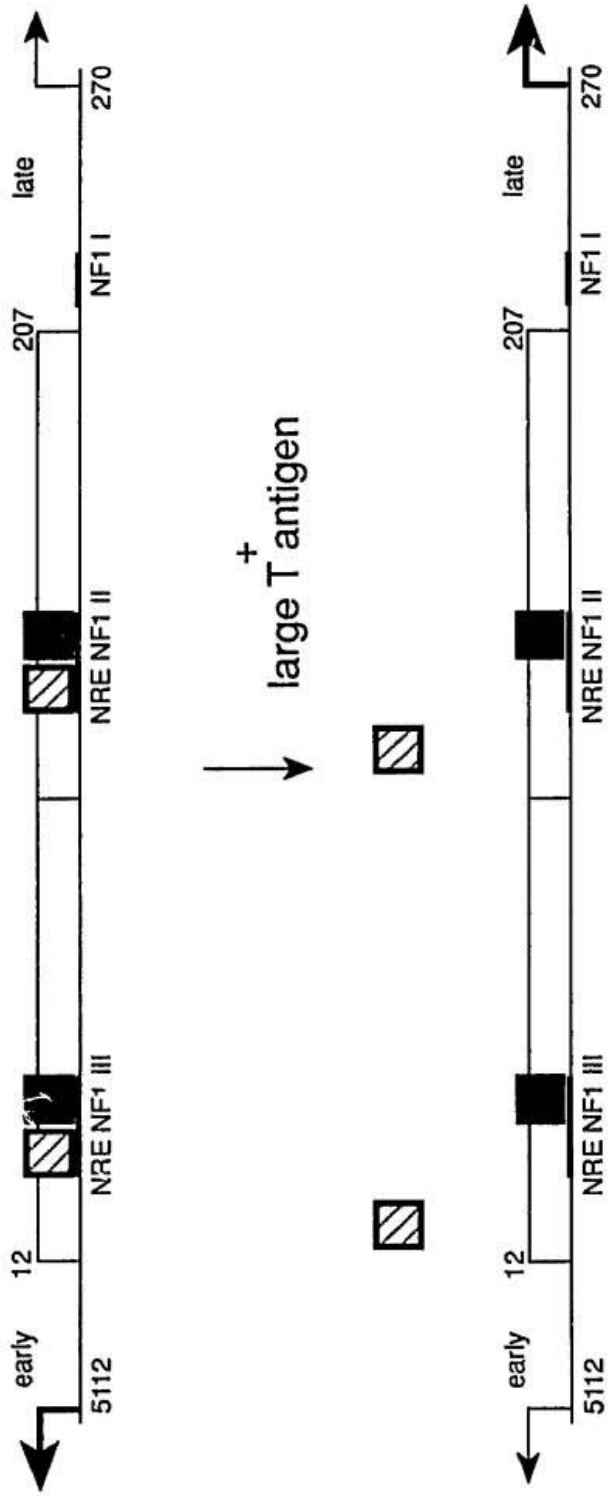
binding of the repressor to NRE.

A single-stranded DNA binding protein named lytic control element-binding protein 1 (LCP-1) binds the lytic control element (LCE) (Tada and Khalili, 1992), previously identified as a pentanucleotide repeat region located adjacent to the NF1 II/III (Tada et al, 1991). Mutations in the LCE affected the binding of LCP-1 leading to reduced early mRNA transcription and had no effect on late mRNA transcription. The possibilities that there may be competition between early and late mRNA start sites for transcription initiation and that occupancy by origin of replication by large T-antigen hinders the early transcription site were ruled out in a study by Khalili et al (1987). It was suggested that JCV T-antigen may inactivate or sequester LCP-1 from binding to LCE/ pentanucleotide repeat region (Tada and Khalili, 1992), analogous to the blocking of binding of AP2 factor to enhancer region of SV40 by large T-antigen (Mitchell et al, 1987). For JCV_L, the 56 kDa protein interacting with LCE/pentanucleotide repeat region might repress the late promoter activity by blocking the interaction of NF1 and TATA box binding factors, which is essential for transcription activation, as confirmed in SV40 system (Gruda et al, 1993).

Based on the results of my studies and those reported by others, I propose a model wherein JCV T-antigen facilitates the increased binding of NF1 II/III by preventing the

Fig. 4.10 Model for possible mechanism of transactivation of JCV late promoter by large T-antigen.

The JCV regulatory region with early and late sides is shown. NRE (pentanucleotide repeat) and NF1 denote the binding sites for negative regulatory element and nuclear factor 1, respectively. The hatched box denotes the repressor protein which binds NRE. The black box denotes the factor binding to NF1 motif. NRE is also called LCE.


early
5112
12
NRE NF1 III
NRE NF1 II
207
NF1 I
late
270
large T antigen
+
early
5112
12
NRE NF1 III
NRE NF1 II
207
NF1 I
late
270

repressor binding to LCE. This would facilitate the interaction of NF1 with NF1 II/III (Fig. 4.10). Thus, the specific interaction of JCV NF1 II/III motifs and brain-specific NF1 may be a doubly important factor in the requirement for NF1-dependent function of the JCV early and late promoter-enhancer in glial cells.

The early promoter of JCV is repressed by JCV large T-antigen (Nakshatri et al, 1990a). Moreover, the integrity of the pentanucleotide repeat region that interacts with the repressor was shown to be important for efficient expression of early but not late promoter-enhancer (Kumar et al, 1992; Tada et al, 1991). My proposed model explains how this could happen. The prevention of repressor binding by T-antigen would lead to activation of late and repression of early promoter of JCV.

CHAPTER 5

Role of cyclic AMP response element (CRE) in JCV expression

5.1 Introduction

The regulatory region of JCV consists of several protein binding domains (reviewed in Frisque and White, 1992; Major et al, 1992; and Fig. 1.2). Site-directed mutagenesis of JCV control region suggested a role for nuclear factor 1 (NF1) binding sequences located in the 98 bp repeats in the glial cell-specificity (chapters 3 and 4 of this thesis). Mutations in all three potential NF1 binding sites greatly reduced the transcriptional activity of JCV early promoter-enhancer in glial cells. However, a residual activity, more than the basal level activity of the pSV0cat vector was still observed, indicating the possible involvement of other factors in expression of JCV (Fig. 3.2). Earlier studies (Amemiya et al, 1992; Ault and Stoner, 1993) had identified a cyclic AMP response element (CRE) in the JCV regulatory region. Examination of the JCV promoter-enhancer sequences revealed the presence of CRE motif, TGAGCTCA 4 bp upstream of the NF1 II and III binding sites, relative to the early region, in the repeats.

Several eucaryotic cellular and viral genes contain cAMP response element (CRE) in their noncoding region and the genes are regulated by cAMP (reviewed in Roesler et al, 1988; Montminy et al, 1990; Borrelli et al, 1992). Analysis of these

sequences identified TGACGTCA as the CRE consensus sequence. A 43 kDa protein called cAMP response element binding protein (CREB) binds this sequence as a dimer. Dimerization and the transcriptional efficiency of CREB are regulated by its phosphorylation by protein kinase A, PKA (Yamamoto et al, 1988). PKA is located in the cytoplasm in an inactive form and as a tetramer consisting of two catalytic subunits complexed with two regulatory subunits. In the presence of elevated intracellular cAMP levels, the catalytic subunits of PKA are activated and translocated to the nucleus, where they phosphorylate CREB on a serine residue. The phosphorylated CREB is then able to bind the CRE sequence of the cAMP responsive genes and activate their transcription (Borrelli et al, 1992). The presence of a CRE motif in the JCV transcriptional control region prompted me to investigate the role of this sequence, in the context of the NF1 site, on the expression of JCV early promoter-enhancer (JCV_E) in glial cells. Attempts were also made to characterize the protein(s) binding to the CRE motif by in vitro binding studies.

5.2 Results

5.2.1 Role of CRE in *in vivo* expression of JCV

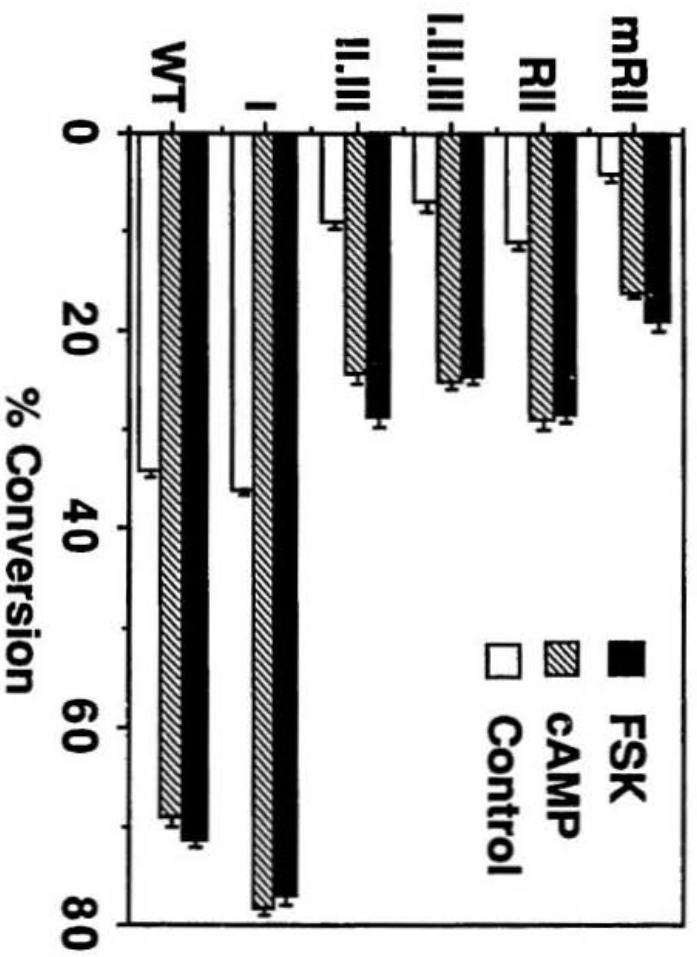
To examine the role of CRE in JCV expression, the plasmids pJC_Ecat for CAT expression from JCV_E and pRIIcat for expression from JCV_E single 98 bp sequence (Fig. 3.1) were transfected into P19 glial cells. The cells were then treated with and without 10 μM of dibutyryl cAMP, a cAMP analogue, for 24 hours. Transfected cells were also treated with 100 μM forskolin, an agent that elevates the intracellular cAMP levels. Both plasmids expressed efficiently in P19 glial cells and treatment with cAMP induced CAT expression from $pJCV_E$ cat and pRIIcat by 2.0- and 2.7-fold, respectively. Forskolin treatment increased expression of these plasmids by 2.1- and 2.6-fold, respectively (Fig. 5.1). No induction of pJCEcat and pRIIEcat by cAMP was observed in UD and DMSO-differentiated P19 cells treated with cAMP and FSK (Fig. 5.2 A and 5.2 B). Thus, the results revealed the functional role of CRE in cell-specific expression of JCV in RA P19 cells.

5.2.2 Effect of NF1 mutations on induction by cAMP of JCV promoter-enhancer

I also wished to examine the effect of mutations in the NF1 binding sites, which are adjacent to the CRE in the 98 bp repeats on the induction by cAMP of JCV promoter-enhancer. Thus, several combinations of NF1 mutants were tested for their expression in the presence or absence of either dibutyryl

Fig. 5.1 Induction by cAMP of CAT expression from JCV_E and the single JCV 98 bp sequence for wild type and mutated NF1 sites in RA-differentiated P19 cells.

Wild type (WT) and mutated CAT expression plasmids are indicated as in Fig. 3.1. Labels: cAMP, cyclic AMP; FSK, forskolin. Values for JCV expression (% Conversion) were normalized with assays for enzyme activity of the RSV-promoter-β-galactosidase plasmid. Results are averages of three experiments.


mRII
RII
I.II.III
II.III
I
WT
0
20
40
60
80
% Conversion
FSK
cAMP
Control

cAMP or forskolin. The mutations in NF1 I introduced into pJCVEcat behaved like wild type with 2.2-and 2.1-fold increased CAT activity in cAMP- and FSK-treated cells, respectively. However, mutations in NF1 II and III (II.III), in all three NF1 binding sites (I.II.III) or in the single 98 bp repeat (mRII) resulted in 2.7-, 3.6-, and 4.2-fold activity, respectively, in cAMP-treated cells. The increase in FSK-treated cells was 3.2-, 3.5-, and 4.8-fold respectively. The increase in activity was more than that observed for WT, I, and RII in cells treated with these agents (Fig. 5.1). In cAMP- and FSK-treated UD and DMSO-differentiated P19 cells, no induction of mutated NF1 CAT expression plasmids by cAMP was noticed (Fig. 5.2 A and 5.2 B). The results suggest that induction by cAMP of JCV_E is more pronounced in an NF1 II and III mutation background in glial cells.

5.2.3 Role of CRE in <u>in vitro</u> expression of JCV

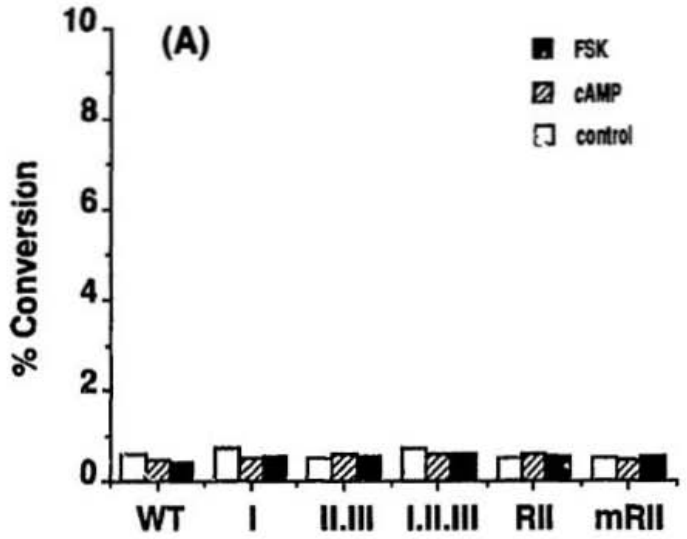
To further substantiate the <u>in vivo</u> observations, <u>in vitro</u> transcription assays were done with RII and mRII CAT expression constructs as described in Fig. 3.1. The RII showed 2.4-fold more activity in FSK-treated cells than in RA-treated P19 cells. Competition with 250-fold excess of either NF1 II/III or CRE oligonucleotides resulted in approximately 50% reduction in activity (Fig. 5.3, RII). The results suggested that the effects of NF1 II/III and CRE binding sites on the expression of JCV_E are additive. The mutation in NF1 II

Fig 5.2 A. Effect of cAMP on CAT expression from JCV_E and JCV 98 bp repeat for wild type and mutated NF1 site in undifferentiated (UD) P19 cells.

Wild type (WT) and mutated CAT expression plasmids are indicated as in Fig. 5.1. Labels and methods are as described for Fig. 5.1. Results are averages of two experiments.

Fig. 5.2 B. Effect of cAMP on CAT expression from JCV_E and JCV 98 bp repeat for wild type and mutated NF1 site in DMSO-differentiated P19 cells.

Plasmids are same as indicated in Fig. 5.1. Methods and labels are as described for Fig. 5.1. Results are averages of two experiments.


(A)
FSK
cAMP
control
% Conversion
10
8
6
4
2
0
WT
I
II.III
I.II.III
RII
mRII

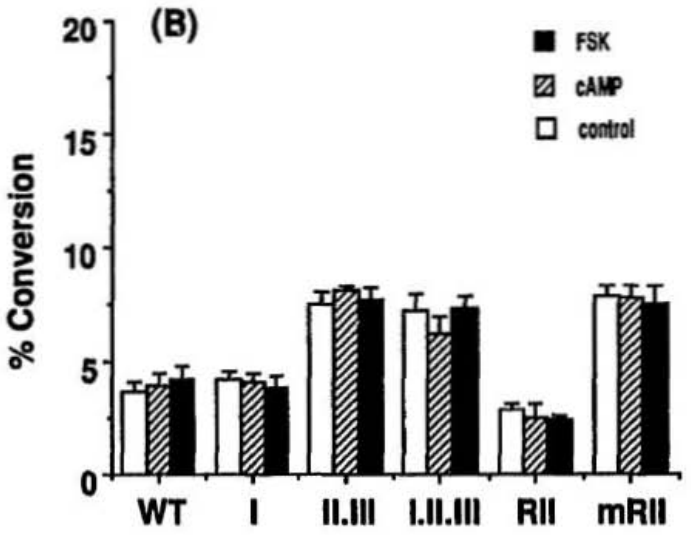
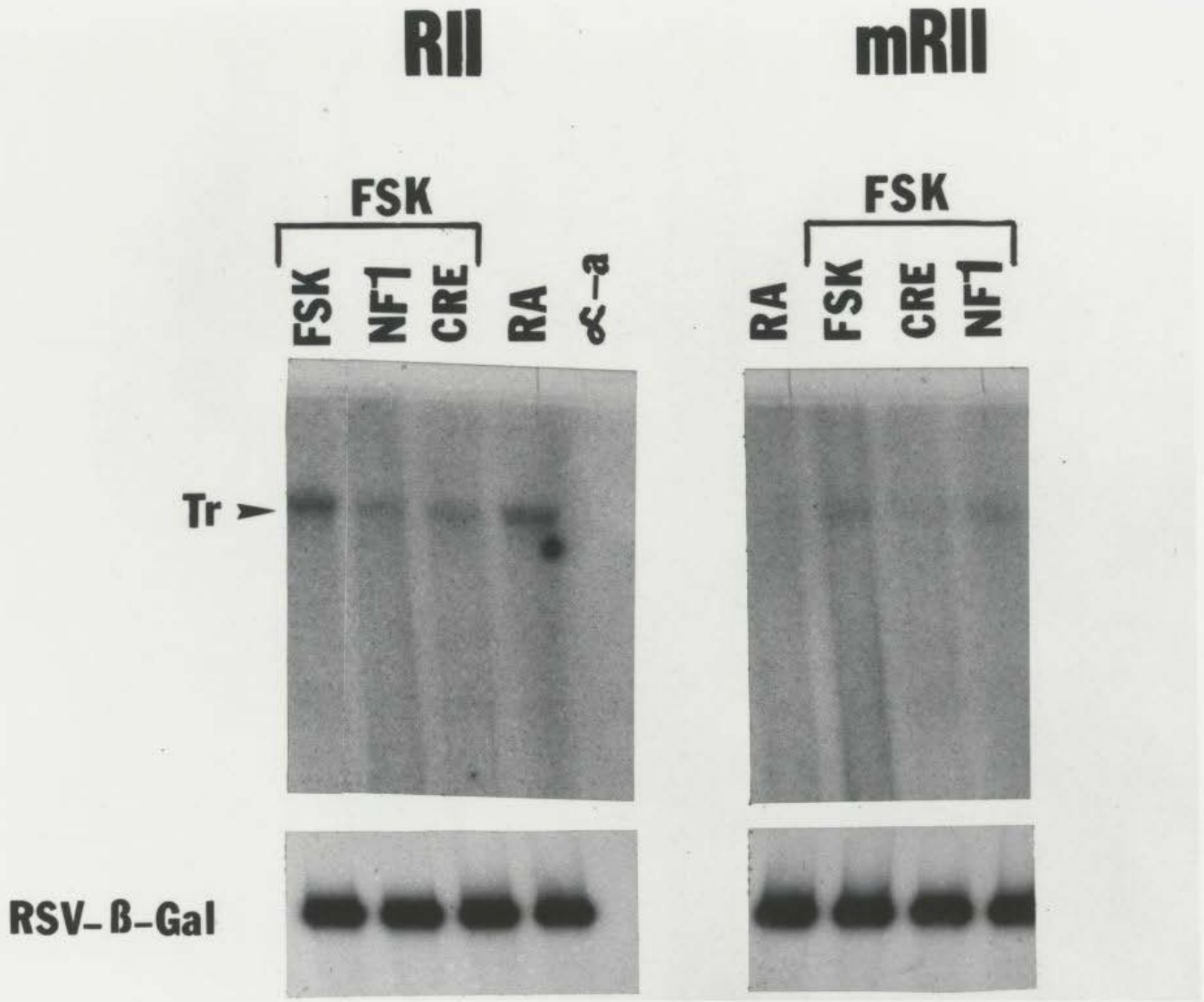

(B)
FSK
cAMP
control
% Conversion
20
15
10
5
0
WT
I
II.III
I.II.III
RII
mRII

Fig. 5.3 <u>In vitro</u> **transcription assays with glial cell extracts and effect of NF1 II/III mutations for cAMP induction.**

RII and mRII are as described in Fig. 3.1. The assays had 60 μg of protein. Labels are FSK, extract from forskolin-treated P19 glial cells; RA, extract from control P19 glial cells; NF1, specific competition by NF II/III oligonucleotide; CRE, competition by JCV CRE oligonucleotide; α-a, 1 μg/ml alpha-amanitin polymerase II inhibitor; Tr, CAT RNA from RII and mRII CAT expression vectors; RSV-β-gal, β-galactosidase internal control RNA. Assays and calculations were as described for Fig. 4.4.


RII
mRII
FSK
FSK
FSK
NF1
CRE
RA
α-a
RA
FSK
CRE
NF1
Tr
RSV-ß-Gal

(mRII) abolished the activity of JCV early promoter in P19 glial cells. In FSK-treated cells, a residual activity was noticed. Competition with 250-fold excess of CRE but not NF1 II/III, oligonucleotide reduced its activity. (Fig. 5.3, mRII). Taken together, the results of in vitro transcription support the in vivo observations. The results also suggest that the increased expression of JCV early promoter-enhancer upon forskolin treatment is due to increased level of CAT transcripts.

5.2.4 Mechanism of cAMP regulation of JCV_E

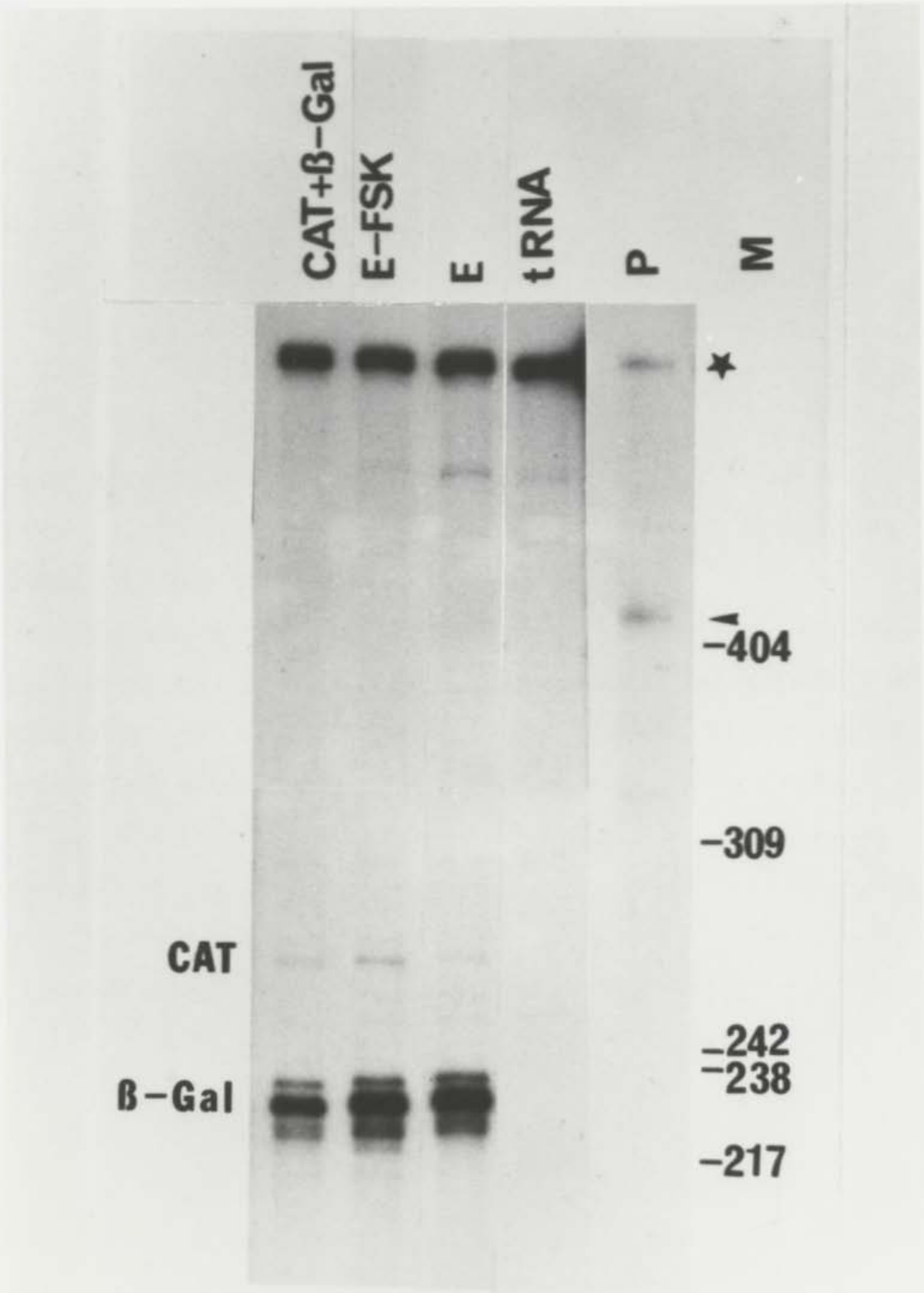
To examine if the increased expression of $pJCV_E$cat was at the level of transcription, RNase protection experiments using RNA from FSK-treated and untreated transfected cells were undertaken (Fig. 5.4). Compared to untreated transfected cells, the transfected RA-treated P19 cells treated with FSK had 2.9-fold more CAT transcripts, as measured densitometrically. These results suggested that the increased expression of $pJCV_E$cat was due to increased levels of CAT transcripts and that the effect of cAMP on the expression of JCV_Ecat is most probably at the level of transcription. This result also confirmed that the in vitro transcription was a reflection of in vivo function.

5.2.5 Effect of mutations in CRE on cAMP induction of JCV promoter-enhancer

To examine the functional role of CRE in JCV expression,

Fig. 5.4 Increased RNA expression from JCV_E after induction by cAMP.

Lanes are: P, probe markers for δ-gal (asterisk on the right) and CAT RNA (arrowhead on the right); CAT+δ-gal, RNA from pRSV-cat- and pRSV-δ-galactosidase-transfected P19 glial cells serving as positive control; E, RNA from control P19 glial cells transfected with pJCEcat; E-FSK, RNA from forskolin-treated P19 glial cells transfected with pJCEcat; tRNA, E.coli tRNA serving as negative control. CAT and δ-gal RNA fragments are indicated on the left and represent RNase T1 resistant products. Molecular weight markers (M) in bp are indicated on the right. Quqntitative estimate of the CAT transcripts in FSK-treated and untreated cells was determined densitometrically and normalized for β-gal transcripts. E, 0.9 and E-FSK, 2.62.


CAT+ß-Gal
E-FSK
E
tRNA
P
M
-404
-309
CAT
-242
-238
ß-Gal
-217

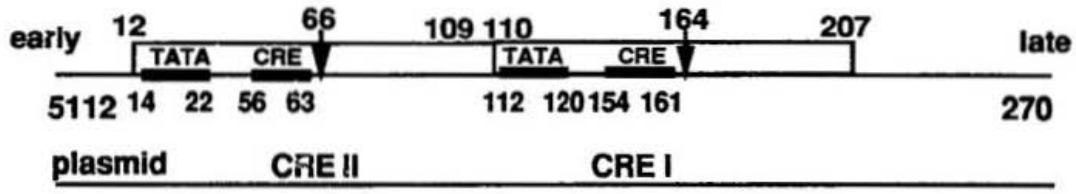
site-specific mutations in CRE were generated by site-directed mutagenesis of JCV promoter-enhancer (Fig. 5.5). The enhancer fragments containing mutations were inserted into pSV0cat. Plasmids were then tested for their functional activity in dibutyryl cAMP-, FSK-treated and untreated P19 glial cells. Wild type whole enhancer (pJCEcat) and single 98 bp repeat (pRIIJCEcat) constructs expressed efficiently in untreated P19 glial cells. Treatment with cAMP resulted in increased expression from pJCEcat and pRIIJCEcat by 2.1- and 3.0-fold, respectively (Fig. 5.6, WT and pRII). Forskolin treatment increased expression of these plasmids by 2.3- and 3.2-fold, respectively (Fig. 5.6, WT and pRII). Mutations in CRE of both repeats or in a single 98 bp repeat revealed no change in the promoter-enhancer activity in either the presence or absence of cAMP or FSK (Fig. 5.5 and 5.6, I.II and mRII). Moreover, the activity of CRE mutants is the same as that of WT constructs in untreated P19 glial cells (Fig. 5.6). These results suggest that the integrity of the CRE sequence is essential for induction of JCV promoter-enhancer by cAMP.

5.2.6 Induction by cAMP of expression of a heterologous promoter in glial cells by cAMP response element (CRE) sequences of JCV

To further examine the functional significance of the CRE motif, a double stranded oligonucleotide corresponding to nt -154 to -161 of JCV_E was cloned (pJCCREcat) in the early

Fig. 5.5 Wild type and mutated CRE motifs in JCV_E sequences in CAT plasmids.

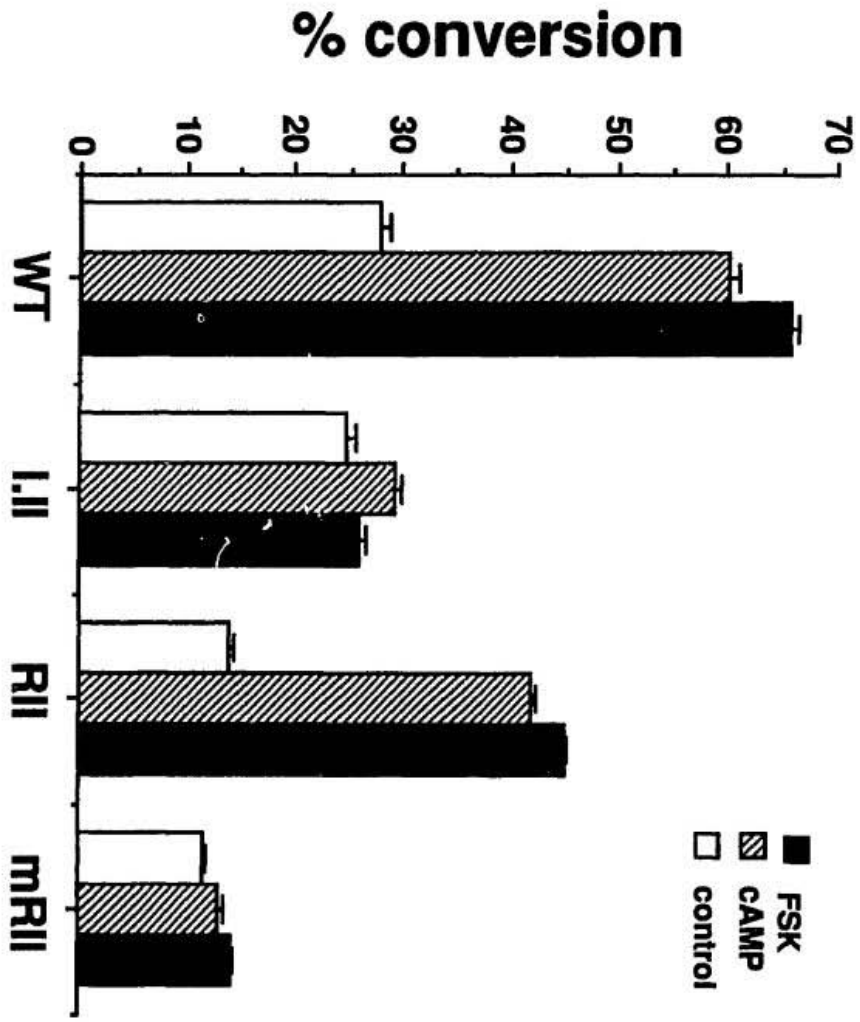
CRE mutations were introduced by site-directed mutagenesis for CAT expression vectors. Top, diagram of sequences present in CAT expression plasmids. Early and late sides are indicated. TATA boxes and CRE motifs are indicated and delineated by thick lines. Nucleotide positions are given below and above the lines. Boxes indicate the 98 bp repeats. Bottom, list of mutated plasmids and sequences for CRE motifs. CRE II and CRE I indicate the CREs adjacent to NF1 III and NF1 II, respectively, in the 98 bp repeats. Mutated CRE nucleotides are indicated by lower case letters and wild type CRE nucleotides are indicated by capital letters.



pJCEcat (WT)	TGAGCTCA	TGAGCTCA
pmJCEcat I.II	TaAGCTCg	TaAGCTCg
pRIIJCEcat (WT)		TGAGCTCA
pmRIIJCEcat		TaAGCTCg

Fig. 5.6 Effect of mutation of JCV CRE motifs on *in vivo* activity.

Results are for CAT assays in untreated control, cAMP- and forskolin (FSK)-treated P19 glial cells. Mutations in CRE motifs are indicated by the unique numbers and letters for the names of plasmids of Fig. 5.5. The cells were transfected 8 hours after being plated with 5 μg of test CAT expression plasmid and 15 μg of pUC19 DNA. The other methods are as described for Fig. 3.2 and 5.1.


% conversion
0
10
20
30
40
50
60
70
WT
I.II
RII
mRII
FSK
cAMP
control

orientation in front of the thymidine kinase (TK) promoter of pBLcat2. The CAT activity of pJCCREcat and the parent vector, pBLcat2, was compared in control untreated and cAMP- and forskolin-treated P19 glial cells. The results are shown in Fig. 5.7. The plasmid pJCCREcat was induced 6-fold in cells treated with either cAMP or FSK. Induction was reduced to 2.6- and 1.5-fold when pUC19 plasmid containing a double stranded JCV CRE oligonucleotide was cotransfected with pJCCREcat in 4- and 8-fold excess amounts, respectively (Fig. 5.7).

To further examine CRE induction, *in vitro* transcription assays were performed. No activity was observed with pBLcat2 template when extract from FSK-treated P19 glial cells was used (Fig. 5.8, pBLcat2, FSK). With pJCCREcat template, no detectable activity was observed with extract from untreated P19 glial cells (Fig. 5.8, pJCCREcat, RA). However, a substantial amount of transcriptional activity was observed with this template and extract from FSK-treated P19 glial cells. This activity was abolished in the presence of 250-fold excess of CRE oligonucleotide (Fig. 5.8, pJCCREcat, CRE). In the presence of 1 μg/ml concentration of alpha-amanitin the transcriptional activity of pJCCREcat was totally abolished, suggesting that transcription was by RNA polymerase II. The results confirmed the presence of a functional CRE sequence in JCV promoter-enhancer. These results also suggested that the JCV CRE sequence enhances the

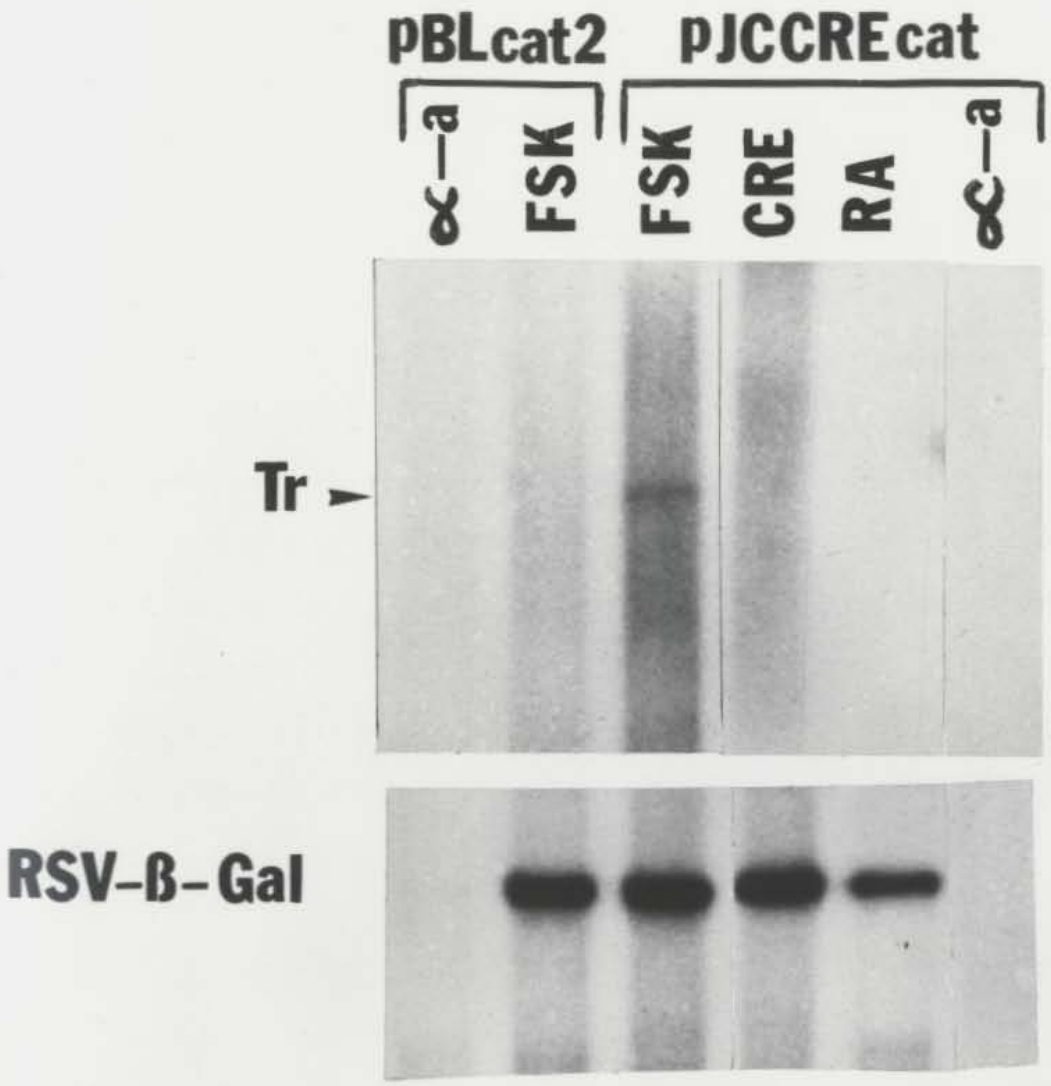
Fig. 5.7 Induction by cAMP of JCV CRE in the context of a heterologous promoter in glial cells.

The vector, pBLcat2 and pJCCREcat are indicated. The 4X and 8X indicate competition with 4- and 8-fold JCV CRE oligonucleotide in plasmid pUC19. Other labels and methods are as described for Fig. 5.1.

pJCCREcat
pBLcat2
8X
4X
FSK
cAMP
Control
0
10
20
% Conversion

Fig. 5.8 Role of cAMP in activity of pBLcat2 and pJCCREcat as assayed by in vitro transcription.

The DNA templates used are indicated on top. Labels are: α-a, 1 μg/ml alpha-amanitin polymerase II inhibitor; Tr, CAT RNA from pJCCREcat and pBLcat2 CAT expression vectors. RSV-β-gal, β-galactosidase internal control RNA. FSK, extract from forskolin-treated P19 RA cells. RA, extract from P19 RA cells. CRE, competition in the presence of 250-fold excess of CRE oligonucleotide.


pBLcat2
pJCCREcat
∝—a
FSK
FSK
CRE
RA
∝—a
Tr
RSV-ß-Gal

transcriptional activity of a heterologous promoter in glial cells.

5.2.7 Interaction of proteins from forskolin-treated P19 glial cells with JCV CRE sequence

5.2.7.1 *In vitro* interactions detected in mobility shift assays

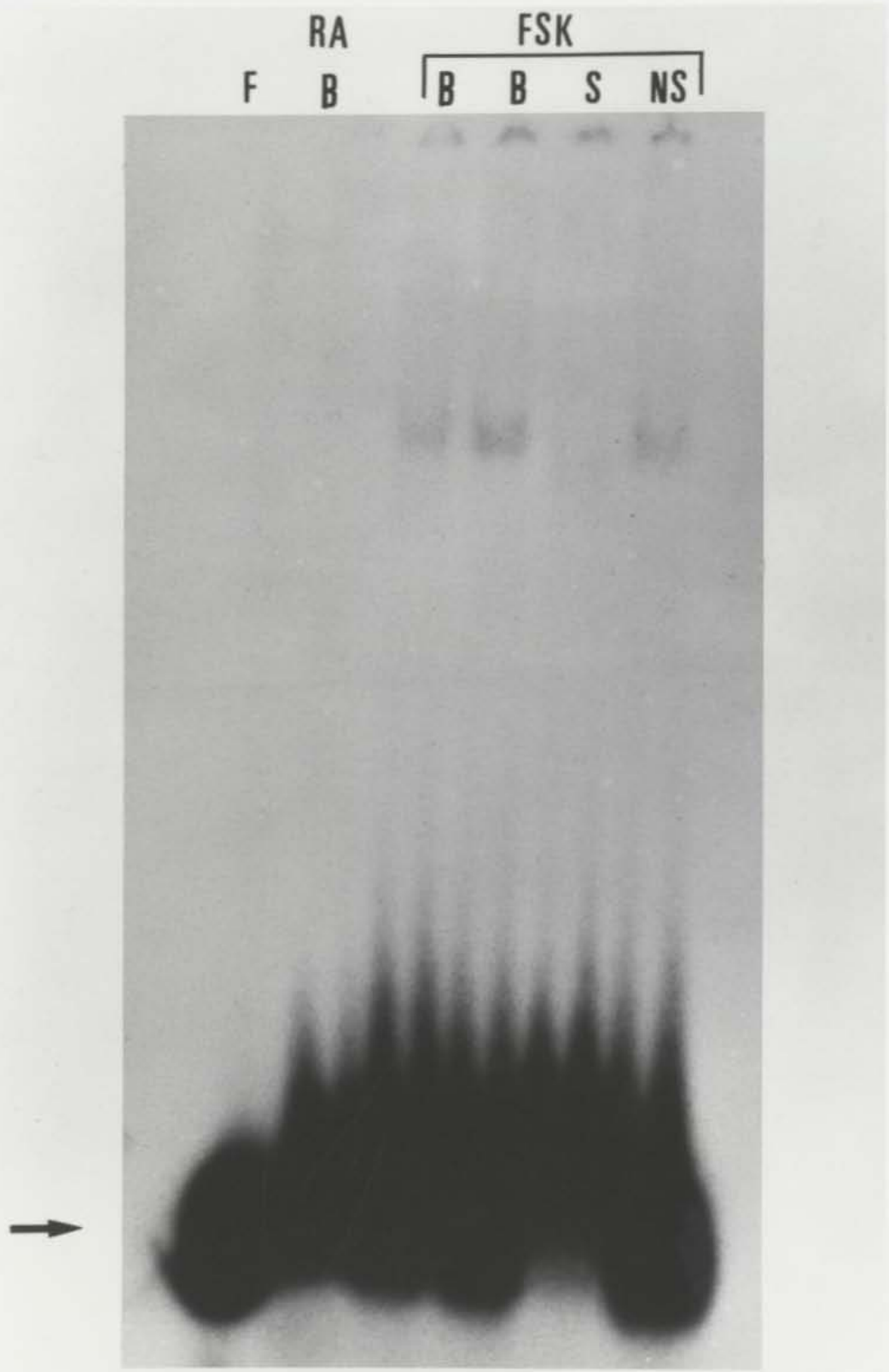
The interaction of a protein with JCV CRE sequence was suggested by the *in vivo* competition with CRE sequence in FSK-treated cells (Fig. 5.7). To examine this possibility, mobility shift experiments with a double stranded oligonucleotide corresponding to JCV CRE sequence was undertaken. The extract from P19 RA cells showed no binding (Fig. 5.9, RA, B). However, with extract from FSK-treated P19 glial cells, one complex was noticed (Fig. 5.9, FSK, B). Addition of 500-fold excess specific competitor CRE sequence abolished the complex (Fig. 5.9, S). Addition of 500-fold excess of a non-specific oligonucleotide (corresponding to NF1 binding sites) had no effect on complex formation (Fig. 5.9, NS). The results suggest that a protein from FSK-treated P19 RA cells, but not untreated cells, specifically interacts with JCV CRE sequence.

5.2.7.2 Identification of protein(s) binding to JCV CRE sequence as determined by Southwestern blot and UV crosslinking assays

To characterize the protein(s) binding to the CRE motif,

Fig. 5.9 *In vitro* binding of JCV CRE oligonucleotide in mobility shift assays.

Lanes are: F, free oligonucleotide probe in the absence of extract; B, binding assay with and no competitor; RA, P19 glial cell extract; FSK, forskolin-treated cell extract; S, specific competition by homologous competitor. NS, non-specific competition by JCV NF1 II oligonucleotide; arrow, free probe.


RA
FSK
F
B
B
B
S
NS

Southwestern blot and UV crosslinking assays were performed. Southwestern blot analysis re-examines the interaction of protein(s) with the oligonucleotide sequence. The Southwestern blot results indicated that a protein of 43 kDa from forskolin-treated P19 glial cells specifically interacts with the CRE sequence (Fig. 5.10, FSK). No interaction of protein(s) from untreated P19 glial cells was observed (Fig. 5.11, RA, B). In UV crosslinking experiments, two proteins of approximately 150 and 43 kDa from FSK-treated cells were crosslinked with JCV CRE oligonucleotide (Fig. 5.11, FSK,B). Addition of 500-fold excess of CRE oligonucleotide abolished the band corresponding to 43 kDa but not 150 kDa (Fig. 5.11, FSK, S). Further, in the presence of 500-fold excess of non-specific oligonucleotides corresponding to the NF1 binding site, NS1 and NS2, the 43 kDa band was not significantly affected (Fig. 5.11, FSK, NS1 and NS2). Taken together, these in vitro assays suggested that a protein(s) interacts with the CRE. This protein appears to be an approximately 43 kDa polypeptide from forskolin-treated P19 glial cells which interacts with the JCV CRE oligonucleotide in a sequence-specific manner in two independent in vitro assays.

Fig. 5.10 Southwestern blot analysis with JCV CRE oligonucleotide.

Lanes are as in Fig. 5.9. Numbers on the right indicate the kDa molecular weights of protein markers. Arrow indicates the 43 kDa protein interacting with the CRE sequence.

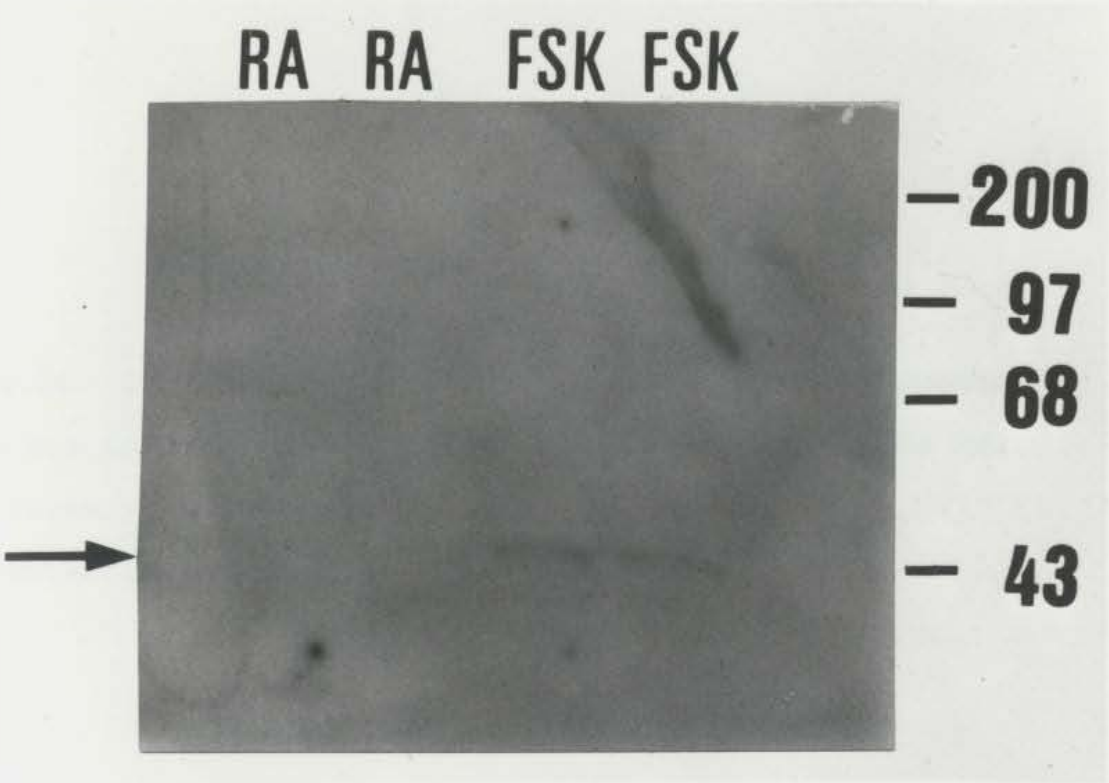
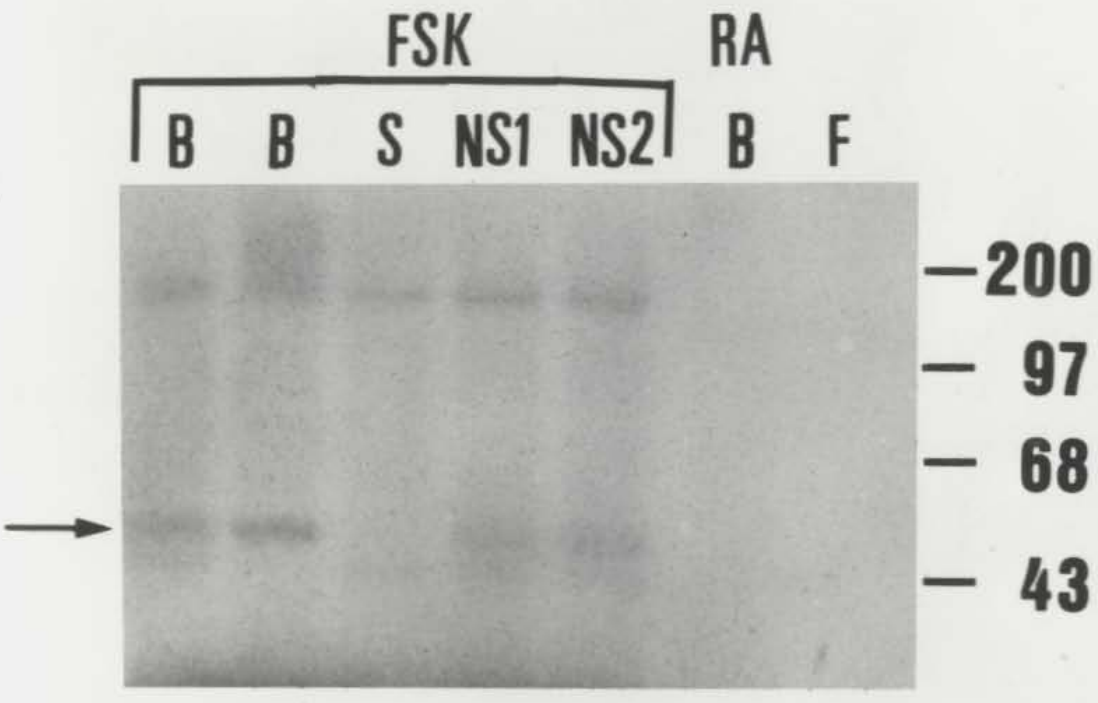

RA
RA
FSK
FSK
200
97
68
43

Fig. 5.11. UV crosslinking assays with JCV CRE sequence. Labels are as described for Figs. 5.9 and 5.10. NS1 and NS2, competition with 500-fold excess of NF1 II and NF1 I oligonucleotides, respectively.


FSK
RA
B
B
S
NS1
NS2
B
F
200
97
68
43

5.3 DISCUSSION

The results of this study clearly demonstrated the presence of a functional CRE in the JCV transcriptional control region. The JCV CRE sequence (TGAGCTCA) is homologous to the consensus CRE (TGACGTCA) except for CG to GC transposition. However, the results of my study suggest without any ambiguity the functional activity of a CRE in the JCV regulatory region. This was based on the observation that the JCV early promoter-enhancer (JCV_E) was inducible by treatment with the cyclic AMP analogue, dibutyryl cAMP, and the cAMP inducer, forskolin. In addition, mutations in the CRE abolished induction of JCV expression by cAMP (Fig. 5.6). The purpose of using forskolin in this study is that forskolin activates the adenylyl cyclase enzyme that converts ATP to cAMP, thus increasing the intracellular cAMP levels. This is followed by activation of PKA which phosphorylates CREB. If there is a functional CRE in JCV_E, the phosphorylated CREB will bind the CRE and activate transcription. The results clearly showed the induction of JCV promoter-enhancer with forskolin, suggesting the existence of a functional CRE in the regulatory region of JCV. In addition, the JCV CRE sequence placed upstream of the TK promoter in pBLcat2 reporter plasmid was inducible in transient cat assays and conferred glial cell-specificity to a heterologous promoter (Fig. 5.7) Increased <u>in vitro</u> transcription was also observed in the

presence of extracts from FSK-treated, but not untreated P19 RA cells (Fig. 5.8). This further confirms the existence of a JCV_E CRE sequence which functions as an enhancer element.

The JCV CRE is located 4 bp from the NF1 binding sites in the 98 bp repeats. Hence, the NF1 mutants were tested for their activity in cAMP- and FSK-treated P19 cells. The results suggest that induction of JCV_E is more on NF1 mutant back ground compared with WT (Fig. 5.1). No induction of JCV_E by cAMP was observed in UD and DMSO-differentiated P19 cells (Fig. 5.2 A and 5.2 B). The results of *in vitro* transcription with RII and mRII suggest that the effects of NF1 binding and CRE motifs are additive.This study was also focused on the characterization of JCV CRE by identifying and characterizing the proteins interacting with CRE.

In previous studies, the understanding of regulation of gene expression by cAMP was largely facilitated by cloning of the cDNAs encoding CRE-binding proteins (CREBs). The cDNAs were initially isolated from rat phaeochromocytoma (PC12) cells and human placental cell libraries (Hoeffler *et al*, 1988; Gonzalez *et al*, 1989). CREB was later shown not to be restricted to these cell types but to be ubiquitously expressed. This suggests a housekeeping role for this factor (Foulkes *et al*, 1991). The fact that CREB has a housekeeping function was demonstrated by using transgenic mice (Struthers *et al*, 1991). The induction of JCV_E by cAMP was observed in

RA-differentiated but not in UD and DMSO-differentiated P19 cells (Fig. 5.1 and 5.2A and B). Since CREB is a ubiquitously expressed factor, the involvement of this protein in glial cell-specific expression of JCV is a puzzle. Sp1, a ubiquitous transcription factor, is developmentally regulated and in mouse and in adult human brains its expression is restricted to oligodendrocytes of the CNS and a limited number of other cell types (Saffer et al, 1991; Henson et al, 1992). It is possible that CREB is developmentally regulated similarly and that different cells contain different forms of CREB.

CREB contains multiple properties that may allow tissue-specific regulation. For example, the transcriptional activation domain of CREB consists of several independent regions. CREB belongs to the leucine-zipper family of proteins (Landschulz et al, 1988). The leucine zipper allows dimerization and formation of the appropriate conformation of the protein. This leads to the interaction of the basic region of the protein with the cognate sequence on DNA. The CREB leucine zipper is important for not only homodimerization but also heterodimerization between two different transcription factors such as CREB and ATF. The heterodimers may have unique cell-specific functions.

The data of JCV in P19 RA cells suggests that the binding of the protein interacting with JCV CRE may be detectable only after phosphorylation of CREB by PKA. In fact,

CREB binding was shown to be modulated by phosphorylation (Yamamoto et al, 1988). Since there are several isoforms of PKA that are tissue specific, it is quite possible that the PKA of P19 RA cells may behave differently (Habener, 1990). Moreover, the consensus phosphorylation sites of various kinases such as protein kinase A (PKA), protein kinase C (PKC), glycogen synthase kinase 3, and casein kinase (CK) I and II present in CREB might be important in regulating its activity. Also, various phosphorylation sites located in CREB cooperate to elicit the regulatory function of the protein (Habener, 1990). In another report, no phosphorylation of CREB was noticed when HeLa cell nuclear extract was used in in vitro studies, although the cells did contain PKA (Merino et al, 1989). This could have been due to lack of certain cell-specific functions. Support for this comes from another interesting finding that PKA affects phosphorylation of CREB variably depending on the cell type (Auwerx and Sassone Corsi, 1991). Thus, the reports indicate that phosphorylation of CREB by PKA depends on the cell type and CREB activity is further regulated by phosphorylation by kinases other than PKA. There also appears to be multiple CREB domains important for homodimerization and heterodimerization which also affect DNA binding and phosphorylation by additional cell-specific functions.

In vitro binding studies have suggested interaction of a 43 kDa CREB protein with CRE (Montminy and Bilezikjian, 1987; Yamamoto *et al*, 1988). There are several proteins belonging to the CREB family and the cDNAs isolated from different tissues suggest that the sizes of the proteins range from 39 to 120 kDa. In my study, the results of UV crosslinking assays revealed the interaction of two proteins of 43 and 150 kDa only in RA P19 cells treated with FSK (Fig. 5.11, FSK, B). In other reports CREB was shown to heterodimerize with other proteins such as ATF, AP2 and c-jun (Borrelli *et al*, 1992). Hence, it is likely that the protein recognizing the JCV CRE sequence binds as a heterodimer. CREB may interact with a factor that is restricted to RA-differentiated P19 cells. Such a factor may be a cell-specific AP2, another protein which requires phosphorylation for efficient DNA binding. The 150 kDa protein was not competed by 500-fold excess homologous CRE oligonucleotide competitor in crosslinking assays (Fig. 5.11, FSK, S). However, CREB was specifically crosslinked with the CRE oligonucleotide. Clarification and use of more sensitive techniques are required. The 43 kDa protein interacting with JCV CRE is the same size as that binding to consensus CRE of the rat somatostatin gene. Whether the protein binding to JCV CRE is the same as the CREB that binds to the consensus CRE remains to be investigated, considering that there are multiple CREB

polypeptides which are also subject to posttranslational modifications.

Southwestern blot analysis with JCV CRE detected only a protein of 43 kDa (Fig. 5.10, FSK). The two techniques are different in their sensitivity and the sequence specificity can better be analyzed with UV crosslinking assays than with Southwestern blots. Moreover, in Southwestern blots the proteins are subjected to renaturation and denaturation, whereas, in UV crosslinking assays the proteins are in their native form. Recently it has been shown that a CREB-binding protein (CBP) of 265 kDa interacts with CREB and may function as a coactivator for efficient transcriptional activity induced by CREB (Chrivia et al, 1993). The existence of such a coactivator in P19 RA cells which cooperates with JCV CRE binding protein for its efficient transcriptional activity remains to be tested.

Interestingly, the human cytochrome P450 C17 promoter (Brentano et al, 1990) and JUN D promoter (Borrelli et al, 1992) contain the TGAGCTC sequence which is 100% homologous to that found in the JCV regulatory sequence. The CREs of these two genes are cAMP inducible and confer cAMP induction potential to a heterologous promoter. Of note, in BK virus belonging to the same family as JCV, a sequence highly homologous to CRE is present 4 bp from the NF1 sites in each 68 bp P block, an arrangement which is identical to JCV DNA

(Deyerle and Subramani, 1988; Cassill et al, 1989). It has been shown that the CRE of BKV is also cAMP inducible (Moens et al, 1990).

Since JCV and BKV share many features, studies with BK virus containing CREs in the transcriptional control region are of interest. Compared to the standard MT-1 BKV strain which has one copy of the CRE motif, the variant with a 34 bp duplication containing the CRE site grew as efficiently as the Gardner strain having three CRE sites. On the other hand, another variant with a deletion in CRE grew poorly in human embryonal kidney (HEK) cells (Sugimoto et al, 1989). It was also shown that insertion mutations in the CRE motif abolished the ability of BKV to transform rat cells (Watanabe and Yoshiike, 1989). Taken together, these studies suggest a functional role for CRE in BKV multiplication and transformation. It will be of interest to isolate JC virus variants which lack CRE sequences or contain more than two copies of CRE. Such variants could be used to study the role of the CRE motif in the multiplication and transformation capabilities of JC virus.

Several genes that are expressed in a tissue-specific manner have been shown to be responsive to elevated cAMP levels. Examples are the phosphoenolpyruvate carboxykinase gene (Chrapkiewicz et al, 1982; Cimbala et al, 1982), the vasoactive intestinal peptide precursor gene (Hayakawa et al,

1984), and the liver tyrosine aminotransferase gene (Hashimoto et al, 1984). Several brain-specific genes possess nuclear factor 1 binding sequences followed by activator prc ein binding sites such as CRE, AP1, ATF, and AP2 (Amemiya et al, 1992). Interestingly, the JCV regulatory region which is strictly glial cell-specific also contains the NF1 binding site with adjacent CRE site. The questions that arise are: "Why does the 98 bp repeat of JCV contain several activator protein binding motifs?" and "What is the significance of the NF1 site and CRE in brain-specific JCV expression?" The in vitro transcription studies with RII and mRII cat constructs (Fig 5.3) preliminarily address these questions. Competition with either NF1 or CRE oligonucleotide reduced the activity of RII to approximately 50% in FSK-treated P19 glial cells. This suggests that both NF1 II/III binding and CRE sequences are important for the increased activity of JCV early promoter-enhancer. In addition, the effects of both of these sites could be additive. This is of interest and consistent with the observation that CRE mutants exhibited similar levels of activity to wild type constructs in untreated P19 glial cells (Fig. 5.6). Since the effects of NF1 and CRE sites are additive, the mechanism by which they activate transcription from JCV_E may be different.

CREB has been shown to interact with several tissue-specific factors. For example, multiple copies of the AP2

protein binding sites placed upstream of the CRE containing genes conferred cAMP inducible transcription, while placing a single copy of the AP2 site had no effect on transcription (Chiu et al, 1987). Forskolin treatment did not increase the binding activity of the AP2, while the transcriptional activity was stimulated, analogous to CREB. The inability of a single but not multiple AP2 sites in activating transcription of CRE containing genes may suggest that AP2 interacts with CREB. Interestingly, using the proenkephalin promoter, it was shown that the AP2 site alone can not confer transcriptional activity but acts synergistically with the CRE to stimulate transcription (Comb et al, 1986). CREB was shown to be expressed ubiquitously whereas AP2 is restricted to certain cell types. AP2 activity is absent in the human hepatoma cell line Hep G2 and in undifferentiated human teratocarcinoma cell line NT2. However, the AP2 activity is induced upon differentiation of the NT2 cells with retinoic acid (Williams et al, 1988). In addition to AP2, CREB was shown to interact with other tissue-specific factors. For example, in case of alpha chorionic gonadotropin, CREB interacts with a protein binding to placental tissue specific element (TSE) adjacent to CRE sites in the promoter (Delegeane et al, 1987). All these studies indicate that CRE binding protein interacts with factor(s) binding to adjacent sites, resulting in efficient transcriptional activity. However, in

my study the results of *in vitro* transcription assays with RII (Fig. 5.3) indicate that the effect of NF1 II/III and adjacent CRE sites on the expression of JCV_E is additive and that the interaction of these factors is unlikely. Most probably, each of these factors stimulate JCV_E expression by different mechanisms. Support for this suggestion are results of experiments with NF1 and CRE mutants (Figs. 5.1, 5.3 and 5.6). While the mutations in NF1 II/III greatly reduced the activity in P19 glial cells, a substantial amount of *in vivo* activity was retained for FSK- or cAMP-treated cells (Fig. 5.1). Moreover, a substantial activity was retained when extract from FSK-treated cells was used for *in vitro* transcription (Fig 5.3, mRII). In addition, the CRE sequence placed upstream of TK promoter of pBLcat2 exhibited *in vivo* activity in the presence of either FSK or cAMP (Fig. 5.7). Also, *in vitro* activity was observed in the presence of extracts from FSK-treated but not untreated P19 RA cells in *in vitro* transcription assays (Fig. 5.8). This confirms that CRE alone can enhance the basal activity of JCV early promoter in glial cells. Overall, the CRE and NF1 II/III sites clearly have a combinatorial effect on the expression of JCV_E and the presence of CRE adjacent to NF1 II/III binding site in the JCV regulatory region further increases its expression in glial cells. This is consistent with the recent speculation that the glial cell-specific expression of JCV early promoter is

regulated by the combinatorial action of transcription factors binding to their cognate sequences, in particular the factors binding to CRE and NF1 sites of JCV enhancer (Henson, 1994).

Brain-specific expression of the JCV_E may be tightly regulated, based on the physiological status of the cell. The CREB protein interacting with CRE motif under conditions with elevated cAMP levels might bind adjacent to the NF1 protein binding to NF1 II/III. This would result in the enhanced expression of JCV early promoter-enhancer. Such a switching mechanism may be regulated by phosphorylation of transcription factors. These factors could tightly regulate JCV_E expression in the NF1 site-dependent, glial cell-specific system . Such a mechanism has been proposed for liver specific gene expression (Roesler et al, 1993).

Glial cell-specific expression of JCV appears to be regulated by both inducible (CREB) and constitutively-produced (NF1) transcription factors. In addition, a motif with extensive homology to the NF-kB sequence of HIV-1 is located outside the regulatory region of JCV. This transcriptional element stimulated the expression of JCV late promoter in glial cells and interacted with PMA inducible and noninducible nuclear proteins from glial cells (Ranganathan and Khalili, 1993). The CRE and NF-kB binding proteins that interact with CRE and NF-kB are coupled to different signal transduction systems via PKA and PKC, respectively. The enhancer-binding

factors that respond to various signal transduction systems may have evolved to couple the transcription of cellular genes to changes in the environment. These changes affect the proliferative state of mammalian cells. Not surprisingly, viruses use the same transcription factors to regulate the expression of their genes, as their life cycles are dependent on the proliferative status of their infected hosts. Thus viruses can respond to different environmental cues by virtue of possessing distinct inducible transcriptional elements in their genome.

NF-kB is sequestered in the cytoplasm by the IkB inhibitor and is released by phosphorylation of IkB by protein kinases such as PKC (Baeuerle and Baltimore, 1988). Cyclic AMP may act as another second messenger leading to NF-kB activation through the activation of PKA (Shirakawa et al, 1989). This might result in the elevation of intracellular cAMP levels, resulting in simultaneous activation of CREB and NF-kB. These would in turn bind to their cognate sequences in the JCV genome, leading to increased transcription of JCV_E and JCV_L. Alternatively, since CREB and NF-kB are activated by two different signal transduction pathways, transcription factors that respond to more than one stimulus may cause a more continuous transcriptional response than those that respond to a single stimulus. This would give the virus a greater advantage of having transcription elements for

response to diverse signal transduction pathways. The identification of the transcription factors that mediate such diverse actions should further our understanding of gene regulation.

CHAPTER 6

FUTURE DIRECTIONS

Tissue-specific gene expression is mainly regulated at the level of transcription. The transcription factors that govern the restricted cell-specific expression of genes are limited to such cells. In chapter 1, I described in detail the transcriptional regulation of cell-specific gene expression. The brain is a complex organ with a heterogeneous population of cells and certain cell types such as glial cells and neurons, allow certain genes to be expressed. This restricted cell-specific gene expression is mainly regulated positively in the cells that are expressed. Expression is negatively regulated in cells in which these genes are not normally expressed.

The understanding of brain-specific gene expression is difficult because of its complex nature. The human JCV polyoma virus is a neurotropic virus and its expression is strictly restricted to glial cells. The regulatory region of JCV contains 98 bp tandem repeats which play a key role in the restricted cell-specific expression. The promoter-enhancer region of JCV contains binding sites for several transcription factors such as nuclear factor 1 (NF1) motifs, pentanucleotide region, polypyrimidine tract and cyclic AMP (cAMP) response element (Fig. 1.2). Several studies used <u>in vitro</u> binding assays and concluded that the NF1 binding sites may play a

role in the glial cell-specific expression of JCV. However, no functional studies were done. My studies presented in chapter 3, clearly demonstrate the importance of NF1 sites located within the repeats in the glial cell-specific expression of JCV. The importance of NF1 binding sites in cell-specific expression of JCV was assessed with JCV promoter-linked reporter genes. In order to understand the effect of mutations in NF1 binding sites in JCV life cycle, it would be important to reconstruct NF1 mutants in JCV whole genome and test their activity in primary human fetal glial (PHFG) cells.

Understanding transcriptional regulation of neurotropic JCV also facilitates the understanding of brain-specific gene regulation. The cDNA encoding the factor binding to the NF1 site in the repeats supported the expression of JCV in non-glial cells, further confirming the importance of NF1 II/III in glial cell-specificity. My studies did not delineate the DNA binding and transactivating domains of the NF1. This can be further studied in the future by functional analysis of exonuclease III deletions of the NF1 cDNA that I isolated. These analyses of the cDNA would also help in understanding the developmentally regulated expression of NF1 proteins in the brain that are important for tissue-specific gene expression, such as the myelin basic protein (MBP) of CNS and the NF1 proteins that activate transcription of liver- and adipocyte-specific gene expression. Whether the gene for the

isolated cDNA is developmentally regulated will be important, because many brain-specific genes contain the NF1 motif (Amemiya et al, 1992). This can be done by Northern blot analysis and RNase protection assays for DMSO- and RA-differentiated P19 cells and for various mouse tissues.

JCV remains latent in kidneys (Frisque and White, 1992) and JCV is capable of replicating in B-lymphocytes (Major et al, 1990). JCV appears to undergo brain-specific types of rearrangements in the lymphocytes, as an intermediate between the kidney and brain. Hence, it will be interesting to study the developmental regulation of NF1 in kidney cells and lymphocytes. Both the MAD strains and archetype strains of JCV can be compared in brain, lymphocytes and kidney tissues.

Replication of the archetype JCV is restricted to the kidney. The hypothesis is that rearrangements in the regulatory region of archetype JCV results in the generation of the brain form of JCV (Ault and Stoner, 1993). Another hypothesis is that JCV remains latent in B lymphocytes and these JCV-infected cells reach the brain, resulting in PML (Dorries et al, 1994). These two possibilities can be tested by looking at the expression of archetype and prototype JCV strains in kidney, B lymphocytes, and brain cells. The site of rearrangements can be checked by Southern blots of DNA isolated from these tissues. This should determine whether the rearrangements in the archetype regulatory region occur in

kidney, in B-lymphocytes, or in brain.

Recently the role of coactivators in the tissue-specific gene expression has gained more attention. For example, the lymphoid cell restricted oct-2, but not the ubiquitously expressed oct-1, was thought allow B-cell-specific gene expression (Gerster et al, 1990). However, recent findings have raised questions about these suggestions. An IgH promoter-specific, oct1 factor-specific and B-cell-specific coactivator, OCA-B, supports the expression of IgH promoter in vitro in the presence of oct-1 but not oct-2 protein (Luo et al, 1992). A coactivator leads to activated transcription by mediating dimerization of liver-specific transcription factor, HNF1 (Mendel et al, 991). A coactivator has also been suggested to help in the communication of basic region of Myo D with its activation domain (Lin and Olson, 1991). Coactivators that support the activated levels of transcription by gene specific regulators such as, Sp1 and CTF/NF1 have also been reported (Pugh and Tjian, 1990). Overall, these observations suggest the role of coactivators in the transcriptional regulation of tissue-specific transcription. Therefore, it is tempting to speculate the requirement for coactivation by an NF1 II/III specific and glial cell-specific coactivator in the glial cell-specific expression of JC virus. Isolation of such a coactivator would also help in the identification of brain-specific coactivators

that participate in activated transcription of brain-specific genes. Affinity chromatography, a powerful tool to identify protein-protein interactions, can be used to isolate the coactivator as described (Luo et al, 1992).

Interestingly, no transcription from the myelin basic protein (MBP) gene was observed in the presence of TFIID isolated from HeLa cells in vitro. However, TFIID extracted from brain cells supported the in vitro transcription of MBP (Tamura et al, 1990a). Therefore, there may be tissue-specific TFIID factors which allow basal level gene expression. The role of such a tissue-specific TFIID in glial cell-specific expression of JCV is an attractive possibility. This can be examined by an affinity assay of extract from P19 cells on an affinity column for TATA sequence of JCV and through an NF1 affinity column.

JCV late promoter is down-regulated by a protein of 56 kDa that interacts with pentanucleotide repeat region located adjacent to NF1 II/III (Tada et al, 1991). However, JCV large T-antigen transactivates the late promoter in glial cells. My results in chapter 4 showed that JCV T-antigen facilitates increased binding of NF1 II/III and thereby activates the late promoter. The results described in chapter 4 lead me to propose a model wherein the T-antigen prevents the binding of a repressor to pentanucleotide repeat region. This would then facilitate the binding of NF1 which is important for

expression of JCV late promoter-enhancer. The isolation of a repressor protein or cDNA encoding this protein would allow this model to be tested. The cDNA library which I made from P19 RA cells and the screening procedure which I used to isolate cDNA encoding NF1 should be of great help. The cDNAs encoding NF1 and repressor can be expressed in E.coli as recombinant proteins and used in vitro for determining their interaction with T-antigen. The isolated NF1 and repressor cDNAs will also be of great help in understanding proteins interacting with one another. This can be achieved by interaction cloning using the yeast two hybrid system (Fields and Song, 1989). Since coactivators form a bridge between gene-specific activators and TBP, the yeast two hybrid system can be used to clone the coactivator.

The adenovirus early gene product, E1a, has been shown to be a potent transactivator (reviewed in Flint and Shenk, 1990). E1a also represses the expression of certain viral and cellular genes (Timmers et al, 1989). For example, E1a blocks the differentiation of muscle cells by repressing the transcriptional activating functions of muscle cell-specific factors such as MyoD and Myf-5 (Braun et al, 1992). I feel that the JCV T-antigen also has a repressor property, analogous to that of E1a. This is based on my observation that JCV T-antigen transactivates JCV late promoter in glial cells, whereas, it represses the same promoter in nonglial cells such

as P19 muscle cells (Fig. 4.2 and 4.3).

Transgenic mice expressing JCV large T-antigen showed regions of demyelination in brain and these regions expressed large T-antigen of JCV. Therefore, my hypothesis is that T-antigen down-regulates the myelin basic protein (MBP) gene leading to demyelination of brain tissue that is observed in PML. Since MBP also contains NF1 binding sequences that are important in its transcription, JCV T-antigen could repress MBP expression indirectly by binding to the MBP promoter through protein-protein interactions. This can be tested by estimating MBP RNA levels by RNase protection assays using RNA from MBP-expressing cells, with and without transfecting T-antigen expression plasmid. If the role of T-antigen in down regulation of MBP is established, regions of the MBP promoter through which T-antigen exerts its repressing effect can be mapped. Another possible mechanism for repression is that T-antigen blocks the differentiation of oligodendrocytes which synthsize myelin. This would be analogous to adenovirus E1a blocking of muscle differentiation and can be similarly tested (Braun et al, 1992).

SV40 small t-antigen has been shown to be involved in enhancing the transcription and transformation capacity of large T-antigen under limiting concentrations of large T-antigen (Bikel et al, 1987). JCV small t-antigen bears high homology to BKV and SV40 small t-antigens. Experiments can be

designed to unravel the role of JCV small t-antigen in the transcription and transformation abilities of JCV.

In chapter 5, I described the identification and characterization of CRE and its functional significance in glial cell-specific expression of JCV. Studies with the BK CRE have shown that CRE plays a role in transformation and multiplication of the virus. I did not perform experiments to determine the importance of JCV CRE in activities other than transcriptional regulation of JCV. Hence, future experiments can be analogously designed to determine whether CRE plays any role in the transformation and multiplication of JCV. In addition, it has been shown previously that NF1 plays a role in the replication of JCV (Sock et al, 1991). My studies have shown that NF1 and CRE binding proteins have an additive effect on transcriptional activity of JCV in glial cells (chapter 5). The possibility that NF1 and CRE binding proteins function in an analogous manner in the replication of JCV remains to be investigated.

Finally, CRE binding proteins are a large family of proteins ranging from 39 to 120 kDa. Since I did not observe cAMP induction of JCV promoter-enhancer in undifferentiated and DMSO-differentiated muscle P19 cells, the protein interacting with JCV CRE probably is a RA P19 cell-specific factor. This factor can now be isolated and characterized by screening the cDNA library from RA-differentiated P19 cells

that I have constructed with Southwestern blots with CRE oligonucleotide.

The RA-differentiated P19 cells contain heterogeneous population of neuronal cells. Since JCV expression is restricted to glial cells only, it is essential to measure the glial cell population in P19 RA cells by using glial cell-specific markers in double immunoflourescence or in situ hybridization techniques. It is interesting to note that RA inhibits nural induction in embryo. However, treatment with 300 nM RA of P19 EC cells induce neural cells in culture. In addition to RA, presence of other factors in whole embryo, but not in RA-treated cells may account for this difference.

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