CHARACTERIZATION OF A cDNA ENCODING A NOVEL TRANSCRIPTION FACTOR REGULATING EXPRESSION OF HUMAN NEUROTROPIC JC VIRUS

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By

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This thesis is dedicated to
my parents

Smt. D. Jayalaxmi
and
Sri. D. Prema Reddy

ABSTRACT

JC virus (JCV) is a ubiquitous human neurotropic polyomavirus and is the etiological agent of the human demyelinating disease, progressive multifocal leukoencephalopathy (PML). Clinically, PML is found when immunodeficiency is caused by neoplasia,chronic disease, the immunosuppressive chemotherapy used for organ transplantation and, more recently, AIDS. JCV is closely related to two other polyomaviruses, BKV and SV40. Unlike BKV and SV40, JCV has a narrow tissue-tropism to glial cells. Several lines of evidence were used to attribute the JCV neurotropism to the viral regulatory region, which is organized as two 98 bp repeats.

Previous studies from our laboratory demonstrated glial cell-specific expression of JCV in differentiating P19 embryonal carcinoma cells. The specificity was shown to be conferred by the NF-1 motifs present in the JCV regulatory region (Nakshatri et al., 1990a; Kumar et al., 1993). A cDNA encoding a factor that binds to the JCV NF-1 motif was cloned from a P19 glial cell cDNA library (Kumar, 1994).

The objective for this thesis was to characterize this cDNA to understand its role in the expression and lifecycle of JCV. First, the cDNA sequence was determined. The analysis of the sequence of the cDNA revealed a significant homology both at protein and nucleic acid levels to a recently characterized

factor interacting with Bcl-2 proto-oncogene protein called BAG-1. However, the cDNA isolated in our laboratory has a different 3'-end (nt 710-973). Therefore the cDNA was named bag-2. The 3'-end of the cDNA appeared to be alternatively spliced, because the point of divergence of the cDNA sequence from that of bag-1 was a consensus splice site.

The bag-2 cDNA encoded a major 30 kDa protein, as determined by in vitro translation. The in vitro-translated BAG-2 protein was bound specifically to the JCV NF-1 II/III oligonucleotide and to the JCV enhancer, as determined by mobility shift and Southwestern blot assays. Further, the transfected bag-2 cDNA specifically transactivated JCV early (JCV_E) and late (JCV_L) promoters in non-glial HeLa cells and the transactivation required the integrity of the JCV NF-1 motifs. The recombinant BAG-2 produced in bacteria stimulated JCV_E transcription in vitro by 3-fold, further suggesting that BAG-2 controls transcription. Interestingly, overexpression of BAG-2 in P19 glial cells inhibited the activated, but not the basal, level of transcription of both JCV_E and JCV_L in a dose dependent manner. However, such an inhibitory effect was not observed in U87 MG human glioblastoma cells. These results are reminiscent of the squelching observed for eukaryotic transcription factors. The bag-1 and 2 mRNAs were expressed in mouse embryonal carcinoma and human cervical cells but not in U87 MG cells. However, the BAG-2 specific C-terminus was expressed only in P19 cells.

Deletion analysis of BAG-2 revealed that the C-terminus (a.a 195-229) was essential for transactivation, while the central α-helical region was important for DNA-binding. Though, the BAG-2 C-terminus was observed in P19 UD, glial and muscle cells, only in P19 glial cells was BAG-2 translocated into the nucleus. This was further confirmed by Southwestern blot analysis with nuclear extracts from these cells.

The BAG-1 is important for modulating cell death. Since BAG-2 showed a significant homology to BAG-1, I tested the effect of BAG-2 on p53 promoter to delineate its possible role in apoptosis. The BAG-2 negatively regulated the mouse p53 promoter, as revealed by CAT assays. Thus, the BAG-2 might protect virus-infected cells from p53-induced apoptosis by decreasing the expression levels of p53. A model was proposed to explain the latency of JCV outside the central nervous system (CNS) and JCV glial cell-specific expression in CNS. In the model, BAG-2 is a novel transcription factor that modulates the expression of JCV differentially in glial and non-glial cells. In the former it activates the expression of JCV as a transcription factor in the nucleus, explaining the neurotropism of JCV. In the latter it inhibits the apoptosis induced by p53 thus, allowing the virus to persist for prolonged time.

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LIST OF ABBREVIATIONS

A	-	Adenine
a.a	-	amino acid position
AdMLP	-	Adenovirus major late promoter
αMEM	-	Alpha modification of Eagle's medium
AIDS	-	Acquired immunodeficiency syndrome
AP-1	-	Activator protein-1
ATF	-	Activating transcription factor
ATP	-	Adenosine triphosphate
BAG-1	-	Bcl-2 associated athanogene-1
Bcl-2	-	B-cell lymphoma/leukaemia-2
BKV	-	BK virus
bp	-	Base pairs
BRL	-	Bethesda Research Laboratories
C	-	Cytosine
CAT	-	Chloramphenicol acetyl transferase
CCG-1	-	Cell cycle gene-1
cDNA	-	Complementary Deoxyribonucleic acid
CIP	-	Calf intestinal alkaline phosphatase
CMV	-	Cytomegalovirus
CNS	-	Central nervous system
c.p.e	-	cytopathic effect
cpm	-	counts per minute
CREB	-	Cyclic AMP response element binding protein

C-terminus	-	Carboxy-terminus
CTD	-	Carboxy terminal domain
CTF	-	CCAAT transcription factor
CTL	-	Cytotoxic T lymphocyte
DAB complex	-	TFIID-TFIIA-TFIIB complex
DB complex	-	TFIID-TFIIB complex
DMSO	-	Dimethylsulfoxide
DNA	-	Deoxyribonucleic acid
DNase I	-	Deoxyribonuclease I
DTT	-	Dithiothreitol
E1A	-	Adenovirus early region 1 A
E1B	-	Adenovirus early region 1 B
EBV	-	Epstein-Barr virus
EC	-	Embryonal carcinoma cells
EDTA	-	Ethylene diamine tetracetic acid
EGTA	-	Ethylene glycol-bis-(β-aminoethyl ether) N,N'-tetracetic acid
G	-	Guanine
GBPi	-	GC-rich binding protein-induced
GFAP	-	Glial Fibrillary Acidic Protein
GTF	-	General transcription factor
HEK	-	Human embryonal kidney cells
HEPES	-	N-(2-hydroxy ethyl) perzine-N'-(2-ethanesulfonic acid)
HIV	-	Human immunodeficiency virus
HNF-1	-	Hepatocyte nuclear factor-1

HPV	-	Human papilloma virus
IE	-	Immediate early
Ig	-	Immunoglobulin
IκB	-	Inhibitor κB
Inr	-	Initiator element
JCV	-	JC virus
JCV_E	-	JCV early promoter-enhancer
JCV_L	-	JCV late promoter-enhancer
kDa	-	Kilodalton
LCE	-	Lytic control element
LCP-1	-	LCE binding protein-1
LTR	-	Long terminal repeat
Mad-1	-	JCV Madison strain-1
MAG	-	Myelin Associated Glycoprotein
MBP	-	Myelin basic protein
MMTV	-	Mouse mammary tumor virus
mRNA	-	Messenger ribonucleic acid
MS	-	Multiple sclerosis
NE	-	Nuclear extract
NF-1	-	Nuclear factor-1
NF-κB	-	Nuclear factor-κB
NP-40	-	Nonidet P-40
nt	-	Nucleotide position
N-terminus	-	Amino-terminus
Oct-1	-	Octamer binding protein-1
ORF	-	Open reading frame

PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PCD	-	Programmed cell death
PCR	-	Polymerase chain reaction
PHFG	-	Primary human fetal glial cells
PLP	-	Proteo lipid protein
PIC	-	Preinitiation complex
PML	-	Progressive multifocal leukoencephalopathy
PMSF	-	Phenylmethylsulfonylfluoride
Pol	-	Polymerase
POU-domain	-	Pit, Oct and UNC-domain
Pur α	-	Purine-rich binding protein α
RA	-	Retinoic acid
RNA	-	Ribonucleic acid
RNAP II	-	RNA polymerase II
RNasin	-	Ribonuclease inhibitor
rpm	-	Revolutions per minute
RSV	-	Rous sarcoma virus
SRF	-	Serum Response Factor
SDS	-	Sodium dodecyl sulfate
SV40	-	Simian virus 40
T	-	Thymine
t-antigen	-	Small tumor antigen
T-antigen	-	Large tumor antigen
TAF	-	TBP associated factor

TAR	-	Tat activating region
TBP	-	TATA box binding protein
TFII	-	Transcription factor II
TGF β	-	Transforming growth factor β
TLC	-	Thin layer chromatography
TNF α	-	Tumor necrosis factor α
Tst-1	-	Testes-specific transcription factor-1
UAS	-	Upstream activating sequence
UD	-	Undifferentiated
USA	-	Upstream stimulatory activity
USF	-	Upstream stimulatory factor
WCE	-	Whole cell extract
YB-1	-	Y-box binding protein-1

CHAPTER 1

INTRODUCTION

1.1 General Introduction

The overall objective of biological research is to understand the basic processes of life. This knowledge can then be applied in learning new strategies for correcting anomalies in the physiological milieu and to control the invasion by microbes, in an effort to preserve homeostasis. "Life" can be defined as a complex mosaic of organ, tissue, cellular and subcellular organization endowed with the propensity of genetic continuity through self-replication. Therefore, the study of viruses lies close to the heart of biology, because the study of viruses not only encompasses the study of life processes but also entwines pathological processes.

Studying viruses to understand the complex process of cell regulation is not an unfamiliar concept. Simian virus 40 (SV40) is an excellent model for studying DNA replication and transcription. Also, the human neurotropic JC virus (JCV) represents a good model to understand brain-specific gene expression. JCV is a member of the polyomaviruses of the family, papovaviridae (Shah, 1990). JCV is the etiological agent for the subacute fatal demyelinating disease called progressive multifocal leukoencephalopathy (PML). The first description of PML was as a "unique and non-classifiable

process", with discrete areas of demyelination and bizarre enlarged astrocytes in two patients (Hallervorden, 1930). Later, PML was described as a distinct clinical entity in chronic lymphocytic leukaemia and Hodgkin's lymphoma patients on the basis of unique pathological features, like extensive demyelination, abnormal oligodendroglial nuclei and giant astrocytes (Astrom et al., 1958).

A viral etiology for PML was suspected when inclusion bodies were detected in the nuclei of infected oligodendrocytes (Cavanaugh et al., 1959). Morphological evidence to further support this observation was first obtained when particles resembling polyomavirus were found in the nuclei of oligodendrocytes with inclusion bodies by electron microscopy (Zu Rhein and Chou, 1965; Zu Rhein, 1967). Subsequently, a polyoma virus was isolated from a PML patient and named JCV after the initials of the patient (Padgett et al., 1971). At the same time, another virus with similar characteristics as JCV was isolated from the urine of a renal transplant patient, and named BK virus (BKV) after the initials of the patient (Gardner et al., 1971). Although both were associated with immunosuppression and found in urine, BKV was never found in the brain of PML patients. Furthermore, BKV was detected more frequently than JCV in the urine (Arthur et al., 1985, 1988; Berger et al., 1987). Although an SV40-like agent was also isolated from the PML patient, the role of this agent in PML pathology was unclear (Weiner et al., 1972).

However, overwhelming evidence now indicates that JCV is the actual etiological agent of PML (Stoner and Ryschkewitsch, 1991; Eizuru et al., 1993).

JC virus is a ubiquitous human symbiote and seroepidemiology studies suggest that the virus is world wide in distribution, with an overall prevalence in adults of 69% (Walker and Padgett, 1983). The spread of JCV is postulated to be by oral or respiratory means (Shah, 1990). Soon after entering the body, JCV remains latent in the kidney (Arthur et al., 1988). This conclusion was based on observations that JCV DNA was detected in the kidneys of normal individuals by DNA hybridization methods (Chesters et al., 1983; Grinnell et al., 1983), suggesting that the kidney is an important site of viral persistence. Persistence represents a latent non-productive infection. Reactivation of JCV is believed to occur under the conditions of immunosuppression observed in bone marrow and renal transplant recipients, in pregnant women, in elderly individuals, in cancer and in AIDS (Coleman et al., 1983; Hogan et al., 1983; Gardner et al., 1984; Arthur et al., 1988; Kitamura et al., 1990). Further support for the above hypothesis comes from the observation that a protein that is inducible by cytokines regulated JCV expression (Raj and Khalili, 1994).

Interestingly, JCV DNA was identified in spleen B cells, bone marrow and brain parenchyma (Houff et al., 1988). This suggests that reactivated JCV may enter the central nervous

system (CNS) via infected B cells and this entry may be the initial event in PML (reviewed in Greenlee, 1990; Major et al., 1992). A haematogenous mode of spread is also supported by the nature of the multifocal demyelinating lesions and their location near the grey-white matter junctions, where the arterioles end (Frisque and White, 1992). Also, the unintegrated form of JCV DNA was detected in liver, spleen, lymph node, and lung to further confirm the haematogenous spread (Grinnell et al., 1983).

Most cases of PML are believed to be the consequence of reactivated infection during immunosuppression. Hence, the appearance of JCV in the AIDS population is relatively frequent. AIDS accounts for nearly 55-85% of the recent cases of PML (Krupp et al., 1985; Berger et al., 1987), supporting the notion that JCV is associated with immunosuppression. Furthermore, direct interaction of a human immunodeficiency virus (HIV) trans-regulatory protein Tat with the JCV regulatory region was demonstrated and this interaction regulated the transcription of JCV (Tada et al., 1990). Recent evidence indicates that 4-5% of patients with AIDS develop PML (Berger et al., 1987; Levy et al., 1988; Quinlivan et al., 1992). Thus, this formerly rare disease, once regarded as clinical curiosity, has lately become remarkably common. PML is the cause of death of 2-4% of AIDS patients in North America (Markowitz et al., 1993).

An association of PML was also observed in lymphatic

leukaemia and Hodgkin's lymphoma (Astrom et al., 1958). Importantly, PML, the once rare disease, is now more common because of widespread use of immunosuppressive chemotherapy in organ transplant recipients (Gardner et al., 1984; Arthur et al., 1988). Recently, JCV was also implicated in the etiology of multiple sclerosis (MS) (Stoner, 1991).

Studies on the neurooncogenicity of JCV are scanty. However, the existing reports demonstrated that the intracerebral inoculation of hamsters with JCV resulted in medulloblastomas, astrocytomas, glioblastomas, peripheral neuroblastomas, meningiomas and retinoblastomas (Walker et al., 1973; Zu Rhein, 1983). A similar inoculation of owl and squirrel monkeys resulted in astrocytomas and neuroblastomas (London et al., 1978, 1983). Thus, JCV, unlike BKV and SV40, is the only polyomavirus that induces tumors in non-human primates. JCV was shown to induce gliomas in PML patients (Sima et al., 1983).

Transgenic mice harboring the JCV early region were developed to study virally-induced tumors and PML. Some animals developed adrenal neuroblastomas which subsequently metastasized to the brain and digestive system (Small et al., 1986b) and some animals suffered from dysmyelination of the CNS but not of the peripheral nervous system (PNS) (Small et al., 1986a; Haas et al., 1994). Further studies have demonstrated that JCV large tumor (T) antigen was expressed mainly in oligodendrocytes of these transgenic mice (Trapp et

al., 1988). The same study also observed the down-regulation of myelin-specific genes, like myelin basic protein (MBP), proteolipid protein (PLP) and myelin associated glycoprotein (MAG). Since T-antigen expression was observed in the cells that produce myelin, it was thought that T-antigen altered the expression of myelin-specific genes and the maturation of oligodendrocytes (Trapp et al., 1988). However, the mechanism of dysmyelination remains unclear.

As a group, polyomaviruses served as models for addressing various biological conundrums, ranging from cancer to the regulation of gene expression. Continued study of the molecular biology of JCV may eventually open new vistas in JCV's PML pathogenicity and neurooncogenicity. Specifically, this knowledge may help in finding a cure or prevention strategy for PML. Generally, the information may shed light on the brain disorders that devastate the lives of 50 million Americans, with an economic impact of $400 billion (Scientific American, 1994).

1.2 JCV

1.2.1 Genome

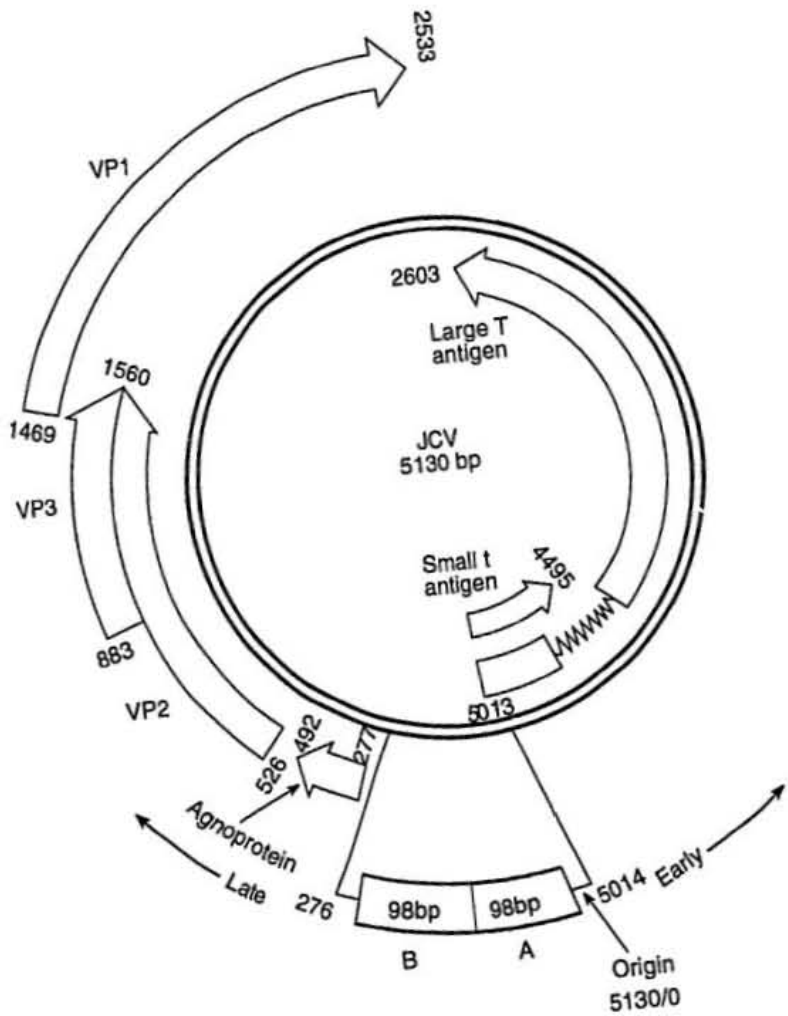
The genome of JCV is a covalently closed circular, double-stranded DNA molecule consisting of 5,130 bp (Fig. 1.1; Frisque et al., 1984). The viral genome codes for two regulatory proteins, T and small tumor (t) antigens; three viral structural proteins VP1, VP2, VP3; and a 7.9 kDa protein

called the agnoprotein since its function is not known. Sequence analysis revealed that the JCV genome was organized as three functional regions. The early region is proximal to the origin of replication, the late region is distal to the origin of replication and the non-coding or regulatory region is located between the early and late regions. The regulatory region contains the promoter-enhancer signals that are important for transcription and the core elements for DNA replication.

The early region codes for two proteins, T- and t-antigens. A single open reading frame (ORF) codes for both T- and t- antigens but an alternative splicing event generates two mRNA species. Therefore, these two proteins have same N-terminal end but different C-terminal ends. The JCV T-antigen has not been studied in detail. Nevertheless, the existing data suggest that it is essential for DNA replication (Lynch and Frisque, 1991), viral-induced cellular transformation (Frisque et al., 1980; Bollag et al., 1989; Haggerty et al., 1989) and trans-regulation of JCV late promoter. The latter determines the transition of transcription from the early to the late stage (Lashagari et al., 1989; Nakshatri et al., 1990a; Tada et al., 1990). Despite 82% homology between JCV and SV40 t-antigens, JCV t-antigen is not effective in enhancing transformation, especially under conditions in which the concentration of T-antigen is limiting (Haggerty et al., 1989).

Fig. 1.1. Circular map of the JCV genome.

The late and early regions are shown in clockwise and anticlockwise directions, respectively. The numbers indicate the nucleotide positions. The locations of core origin of replication and regulatory region are indicated at the bottom. The regions A and B indicate the two 98 bp tandem repeats of the regulatory region. The large open arrows inside the circle indicate the open reading frames of large and small tumor antigens, respectively. The wavy line indicates the intron sequence in the large T-antigen coding sequence. The open arrows outside the circle indicate the open reading frames of viral structural proteins and of agnoprotein. The figure is not drawn to scale.


2533
VP1
2603
Large T
antigen
1560
1469
JCV
5130 bp
VP3
Small t
antigen
4495
883
VP2
5013
492
277
526
Agnoprotein
Late
276
98bp
98bp
5014 Early
B
A
Origin
5130/0

The late region codes for the agnoprotein and three viral structural proteins, namely VP1, VP2 and VP3. The smallest ORF is at the 5' end of the late coding region and encodes the agnoprotein, which may be involved in the viral capsid assembly (Hou-Jang et al., 1987) and has DNA binding capacity (Jay et al., 1981). The largest ORF is at the 3' end and codes for VP1, the major virion structural protein. Sequences between the 5' and 3' ORFs of agnoprotein and VP1 encode VP2 and VP3 (see Fig. 1.1; Frisque et al., 1984). JCV exhibits an extensive 70% sequence homology with SV40 and 75% with human BK virus (BKV) and the homology is weakest in the noncoding region (Osborn et al., 1974; Howley et al., 1976). Unlike BKV and SV40, the JCV Mad-1 strain is strictly neurotropic and replicates efficiently only in cultures which are rich in spongioblasts or astrocytes (Padgett et al., 1977; Major and Vacante, 1989).

Most of the JCV isolates were derived from brain tissues, kidneys and urine of PML patients and a few were from urine of transplant recipients or normal individuals. The first isolation of JCV was reported from Madison, Wisconsin after cultivation of JCV in primary human fetal glial cells (PHFG) (Padgett et al., 1971). It was referred to as the Mad-1 strain and was designated as the "prototype", since no rearrangements were observed in the viral genome during continuous passage in culture. Mad-1 has two 98 bp tandem repeats of regulatory region with a uniquely duplicated TATA box (Martin et al.,

1985). Subsequent isolates were designated Mad-2, Mad-4 etc.

The genomes of JCV from urine of non-PML patients revealed a 23 bp insertion in the regulatory region which is absent in the Mad-1 strain (Martin et al., 1983). A 66 bp insertion was also found in these genomes between nucleotide position (nt) 80 and 98 of the tandem repeats (Yogo et al., 1990). Therefore, the JCV genome with sequence rearrangements containing 23 bp and 66 bp insertions was suggested to be the "archetype" genome. Based on these results, it was suggested that the hypervariability of the JCV genome may be a process of adaption of JCV for replication in the brain (Loeber and Dorries, 1988).

1.2.2 Regulatory region

Though JCV exists in kidneys, replication and expression are only efficient in the brain. This restricted tissue-specificity was attributed to the promoter-enhancer region, because the greatest degree of divergence of DNA organization was in the regulatory region of JCV, compared to other papovaviruses such as BKV and SV40 (Kenney et al., 1984). JCV promoter-enhancer signals were identified, based on sequence comparisons with the BKV and SV40 regulatory regions (Frisque et al., 1984). Sequences between nucleotides 5,014 to 276 represent the non-coding region of JCV. The regulatory region of JCV is bidirectional in function and contains the origin of

replication and *cis*-acting signals for early and late transcription.

The regulatory region of JCV has uniquely duplicated TATA boxes. These two TATA boxes were believed to control early and late transcription of the virus. Some controversy exists regarding the location of the 5' terminus of early mRNAs. Initially, S1 nuclease mapping was used to show that viral mRNA start sites for early gene expression were at nt 122-125 and concluded that the TATA element present in the repeat B, distal to origin of replication functioned as a component of the early promoter (Kenney *et al.*, 1986). Later, transcription initiated at nt 5,112 and 5,082 from the TATA box was attributed by primer extension analysis to the repeat A, proximal to the replication origin but not in repeat B (Khalili *et al.*, 1987). A more recent report resolved the discrepancy by showing that the early and late genes were initiated at nt 5,115-5,125 and 200-203, respectively (Daniel and Frisque, 1993). Taken together at face value, these results suggest that both TATA boxes are utilized for the early and late gene transcription of JCV.

In general, viral enhancers are modular collections of a myriad of *cis*-acting elements that are bound by a variety of cellular transcription factors to enhance and/or repress transcription. Considerable attention was focussed on these elements, because they affect gene expression in a tissue- and species-specific fashion.

1.2.3 *Trans*-acting factors binding promoter-enhancer sequences

Cell culture studies clarified that the glial cell-specific expression of JCV was due to a number of intracellular factors that bind to the promoter-enhancer sequences of JCV (Frisque *et al.*, 1979). Subsequent studies delineated the *cis*-acting DNA signals and their cognate *trans*-acting protein factors.

To identify the cellular proteins that interact with JCV enhancer sequences, UV crosslinking and gel mobility shift assays were employed with HeLa and human fetal brain nuclear extracts and complementary oligonucleotides that span the 98 bp tandem repeats of JCV. Oligonucleotides spanning the nt 134-160 of 98 bp repeat interacted with proteins of 45 and 85 kDa from fetal brain and HeLa cells, respectively. Oligonucleotides having homology to the 5' and 3' terminal regions of the JCV 98 bp repeat sequence interacted with 82 and 78-80 kDa proteins (believed to be a TATA-like factor) from both extracts. A 230 kDa protein which was present in both extracts interacted with several regulatory sequences within the 98 bp repeat (Khalili *et al.*, 1988).

Further, three protected regions were observed using DNase I footprinting assays with mouse brain nuclear extracts; two were within and one was outside the repeats. The two protected sequences within the repeats were shown to be homologous to nuclear factor-1 (NF-1) binding sites (nt 35-58

and 133-156) and the sequences outside the repeat (nt 208-229) were shown to be a pseudo NF-1 motif (Tamura et al., 1988).

Later studies employing mobility shift assays identified binding activities in nuclear extracts from A172 human glioma, PHFG and HeLa cells. No binding was detected with extracts from HEK cells. Furthermore, DNase I footprinting studies were done with the same extracts protected the NF-1 sequences similar to those, identified in the above described studies. Protection of these NF-1 sequences was competed by oligonucleotides homologous to the adenovirus replication origin, which contains NF-1 binding sites (Amemiya et al., 1989). Also, a SacI motif was reported to overlap each NF-1 site present in the repeats. However, the nature of the proteins that bind to this motif was not clarified (Tamura et al., 1990b). Later, it was shown that this region interacted with the cyclic AMP response element (CRE) binding protein (CREB) (Kumar, 1994).

Studies from our laboratory identified three protected regions in the JCV regulatory region by DNase I footprinting assays with nuclear extracts from P19 glial cells. The two protected regions present in the repeats were named region II and III. The protected region outside the repeat was named region I. All these regions contained sequences homologous to NF-1 binding sites (Nakshatri et al., 1990a). Subsequently, the proteins interacting with these NF-1 motifs were characterized in mobility shift assays (Kumar et al., 1993).

(**Note:** The NF-1 motifs in regions II and III are 6 bp inverted palindromic repeats containing the same sequence. Therefore, the JCV NF-1 motif is referred NF-1 II/III motif, throughout the text).

A 45 kDa protein from calf brain that interacted with the JCV B domain (equivalent to NF-1 II/III) was purified by DNA-affinity chromatography from calf brain and was shown to specifically stimulate transcription of JCV_E (Ahmed et al., 1990a). Subsequently, a cDNA was cloned from a human brain cDNA library using domain B as a probe that codes for a 45 kDa glial factor-1 (GF-1), stimulated the transcription of JCV_L and to a limited extent JCV_E (Kerr and Khalili, 1991).

Tst-1, a POU-domain transcription factor family member, was shown to stimulate JCV transcription and DNA replication. Therefore, Tst-1 was suggested to be one of the factors determining the glial cell-specificity of JCV (Wegner et al., 1993). A CRE adjacent to the NF-1 region II/III was speculated (Amemiya et al., 1992). Work in our laboratory showed that a 43 kDa protein interacted with this putative CRE and regulated JCV_E gene expression (Kumar, 1994).

By DNase I footprinting with purified proteins, binding of Jun, a proto-oncogene product to the AP-1 site near the NF-1 region was shown and the binding suggested a direct interaction between Jun and NF-1 proteins bound to their respective DNA sites (Amemiya et al., 1991).

An AGGGAAGGGA pentanucleotide repeat domain, also called

the lytic control element (LCE) (Tada and Khalili, 1992) is present between the NF-1 region II/III and TATA box of each repeat and was shown to have a role in transcription (Tada et al., 1991) and replication (Lynch and Frisque, 1990; Chang et al., 1994). The LCE also binds to a 50-52 kDa single strand LCE binding protein (LCP-1) derived from glial cell extracts (Tada and Khalili, 1992). Also, a 56-60 kDa double-stranded DNA- binding protein from glial and non-glial cell extracts down-regulated JCV_L gene expression (Tada et al., 1991; Sharma and Kumar, 1991).

In the presence of the adjacent NF-1 and TATA motifs this pentanucleotide repeat was shown to interact with a protein of 70-80 kDa. This suggested that the interaction facilitated the transcriptional regulation of JCV_E promoter (Kumar et al., 1994). Interestingly, a cDNA was isolated from a HeLa cell cDNA library, using a B region probe comprising the NF-1 and AGGGAAGGGA motifs (nt 134-160). This cDNA encoded a YB-1 protein that bound to the pyrimidine-rich opposite strand (early strand) of the pentanucleotide repeat in single- and double- stranded conformations (Kerr et al., 1994). The same study demonstrated that Purα and LCP-1 were the same proteins. Subsequent work from the same group showed that Purα (a single-stranded DNA binding protein) interacted with the pentanucleotide repeat of the late strand and activated the transcription of JCV_E (Chen et al., 1995 a and b).

The HIV type 1 (HIV-1) trans-regulatory protein tat was

shown to be a potent activator of JCV late gene expression (Chowdhury et al., 1990; Tada et al., 1990). The tat-responsive region was mapped to nt between 5,112-5,130. Tat responsiveness is mediated by two regions within the JCV_L on either side of the transcription initiation start site (Chowdhury et al., 1993). Tat was shown to interact directly with the JCV RNA transcript containing the downstream region, analogous to its interaction with HIV TAR (Chowdhury et al., 1992).

In another study, two T-antigen binding sites were identified which play a role in JCV DNA replication (Chang et al., 1994). The T-antigen binding site I was identified at the early side of the origin of replication while the T-antigen binding site II was in the origin of replication containing four T-antigen binding sites.

Another study speculated that a transcription factor abundant in B cells binds the Oct-1 motif and the NF-κB binding sites in the JCV enhancer (Major et al., 1990). Subsequent work characterized the NF-κB site present outside the repeats and showed that it regulates the transcription of the JCV_L (Ranganathan and Khalili, 1993).

Domain D on the late side of the JCV regulatory region interacts with 43-50 kDa range proteins from both glial and HeLa cells. This domain activates the early, but not the late promoter of JCV in brain cells (Ahmed et al., 1990b).

Taken together, these data suggest that the cell type-

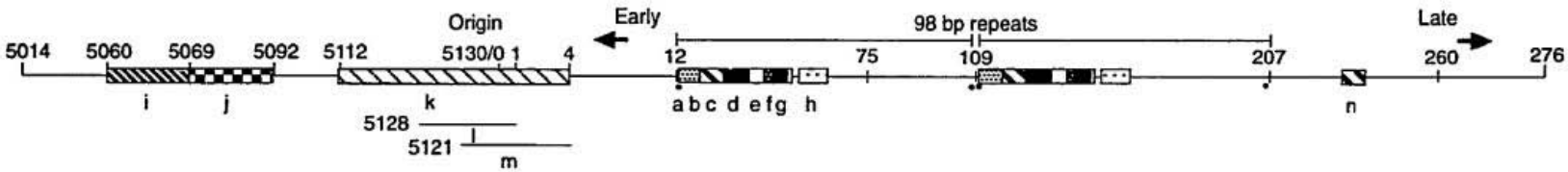
specific expression of JCV is regulated by both positive and negative regulatory mechanisms. Though JCV is present in a vast majority of the population as a latent infection of the kidney, only under immunocompromising conditions does a productive infection occur. However, it is not clear how immunosuppressive conditions would reactivate JC virus. Recently, a study identified a novel 81 kDa protein which was inducible by interleukin-1β, TNF-α, γ-interferon and TGF-β. The 81 kDa protein was bound to a GGA/C-rich sequence in the viral origin of replication and was shown to act as a transcriptional repressor of JCV_L. This novel inducible protein was named GBPi for GC-rich region binding protein inducible. When a change in the cytokine profile occurs, usually under immunocompromising conditions, reactivation of JCV infection follows (Raj and Khalili, 1994; Atwood et al., 1995). Fig. 1.2 summarizes the JCV cis-acting signals and the putative transcription factors.

1.2.4 Restricted growth in glial cell cultures

JCV is strictly a human neurotropic virus. JCV was originally isolated from cultures of PHFG. Enhanced propagation of JCV was observed in these cells at 39^0 C rather 37^0 C (Grinnel et al., 1982). The PHFG cell population is heterogeneous, and most studies have shown that a culture containing a high proportion of spongioblasts, the precursors of oligodendrocytes, was necessary for optimum growth of JCV

Fig. 1.2. JCV regulatory region indicating *cis*-elements and their *trans*-acting factors.

The numbers indicate the nucleotide positions. The *cis*-acting elements are indicated by the lower case letters. The legend below the figure shows the molecular weights and the characteristics of the *trans*-acting factors that interact with the *cis*-acting elements. A detailed description of these *trans*- acting factors is given in the text. The diagram is not drawn to scale.



***Cis*-elements**	***Trans*-acting factors**
a. Tst -1	45 kDa
b. TATA box	
c. Poly A region	78 - 80 kDa
d. LCE	70 - 80 kDa (only in presence of adjacent poly A tract)
	53 kDa (only on early side)
	56 - 60 kDa (only on late side)
	Pur α (single strand DNA, late strand only) 50 - 52 & 100 - 120 kDa
	YB - 1 (single- and double- strand C/T- rich DNA, early strand only)
e. NF- 1	45 - 88 kDa (glial cells)
	65 kDa (P19 RA cells)
	85 - 230 kDa (HeLa cells)
f. GF- 1	45 kDa
g. CRE	43 kDa
h. AP-1	
i. NF-κB	35, >68, 56, 48 & 87 - 102 kDa
j. T antigen site 1	
k. GBPi	81 kDa
l. Tat responsive region	
m. T antigen site 2	
n. Domain D	43 - 50 kDa

(Padgett et al., 1977). Virions were detected in the nuclei of the spongioblasts and rarely in the nuclei of the astrocytes, the second most abundant glial cell population (Padgett et al., 1971). Contrary to these results, another study showed that JCV can be propagated in astrocyte-enriched cultures (Major and Vacante, 1989).

The PHFG cells provided the best system for propagation of JCV, because continuous passage of JCV in these cells produced no changes in the genome, transcriptional activity and length of the lytic cycle of the virus. However, the procurement and cultivation of these cells are difficult. In addition, the JCV cytopathic effect (c.p.e) is subtle. An alternate permissive cell system for JCV propagation was sought for. To this end, primary human embryonic epithelial cells originating from the kidney, lung, liver and intestine, and fibroblasts, were assessed (Padgett and Walker, 1976; Miyamura et al., 1980; Beckman et al., 1982). Although JCV was adapted to propagation in human embryonic kidney (HEK) cells, the DNA found in the virus was defective. Also, the other cell systems were not found to be useful.

Unlike human cells, animal cells were absolutely non-permissive for JCV. So, it was thought that the restricted host range of JCV was at the stages of virus adsorption, penetration or uncoating. To test this hypothesis JCV DNA was transfected into HEK, lung and PHFG cells. Viral DNA was recovered only from the PHFG cells, not the other cell types

(Frisque et al., 1979). Thus, it appeared that the restricted cell-specificity of JCV was due to intracellular factors controlling viral expression and replication (Frisque et al., 1979; Miller et al., 1983).

1.2.5 Latent JCV infection

The cell type in which JCV remains latent after primary infection is elusive. Initially, it was thought JCV could be latent in brain cells. Brain samples from the immunosuppressed and non-immunosuppressed, but not PML individuals, failed to show any signs of viral genomes or viral proteins, either by PCR or immunohistochemistry (Hogan et al., 1980; Arthur et al., 1989; Telenti et al., 1990; Henson et al., 1991). This suggests that JCV remains latent outside the CNS and becomes reactivated in the event of immunosuppression and gains access to the CNS. This reactivation could have two sequelae; one is the lytic infection of oligodendrocytes and the other is viruria, in which infectious virus may be shed in urine (reviewed in Frisque and White, 1992).

The idea that kidney is the main site of JCV latency was based on viruria observed during pregnancy (Coleman et al., 1980), old age (Kitamura et al., 1990), cancer (Hogan et al., 1983), AIDS (Markowitz et al., 1980), and other unclassified conditions (Arthur and Shah, 1989). The JCV genome was reported in 10% of normal kidneys (Chesters et al., 1983). By dot blot hybridization, JCV DNA was also found in the lungs,

livers, lymph nodes and spleens of PML patients (Grinnell et al., 1983).

Interestingly, JCV DNA was also found in B cells in the bone marrow, spleen and brain parenchyma (Houff et al., 1988). The presence of capsid antigens and 200 copies/cell of viral DNA in B cells, suggests that viral replication had occurred. More recently, peripheral blood leucocytes of normal individuals were also shown to harbor JCV, thus providing strong evidence for the persistence of JCV in these cells (Dorries et al., 1994). Taken together, these results suggest that JCV remains latent in B cells of bone marrow, and with the loss of the integrated immune response, such as during cellular immunodeficiency, the B cells may expand. Such activated B cells that aid in the active phase of viral replication and transcription, may enter the CNS by the vascular system and infect the brain (Ault and Stoner, 1994). The other sites of the JCV latency are unknown at this time. However, it is possible that other organs besides B cells and kidney may harbor JCV.

However, the exact mechanism of JCV latency is not known. Several mechanisms have been proposed to explain the latency exhibited by most of the DNA tumor viruses (Ahmed and Stevens, 1990). I would like to limit my discussion to the role of Bcl-2 protein in viral latency, in particular, because this discussion is more pertinent to the results obtained in this study.

Bcl-2 stands for B-cell lymphoma/leukaemia-2 gene. Bcl-2 was first discovered through its involvement in B cell malignancies, where the bcl-2 gene is moved from its normal chromosomal location into juxtaposition with powerful promoters of the Immunoglobulin heavy chain (IgH) gene. Thus, deregulation by the translocated bcl-2 gene lead to the overproduction of bcl-2 mRNAs and their encoded proteins (Tsujimoto et al., 1985; reviewed in Croce, 1987; Nowell and Croce, 1987). Bcl-2 was first shown to prolong cell survival in immature pre-B-cells, where stable transfer of bcl-2 permitted prolonged cell survival even in the absence of lymphokines (Vaux et al., 1988). Since lymphokines prolong the cell survival by preventing apoptosis or programmed cell death (PCD) (Tushinski et al., 1982; Williams et al., 1990), these results suggest that Bcl-2 is capable of blocking PCD, and subsequently this ability was formally demonstrated (Hockenbery et al., 1990). The protein encoded by the bcl-2 gene contains no sequence motifs that might suggest a biochemical function for Bcl-2. The most noteworthy feature is a stretch of highly conserved 19 hydrophobic amino acids at the C-terminus, which serve to anchor Bcl-2 to membranes (Cazals-Hatem et al., 1992; Sato et al., 1994). However, the mechanism of action by which the Bcl-2 protein protects cells from apoptotic cell death is not very clear. For example, in cells protected by Bcl-2 from apoptosis, none of the characteristic morphological changes, such as, cell shrinkage,

chromatin condensation and nuclear fragmentation, were observed. This indicates that Bcl-2 blocks a final common pathway leading to apoptosis. The participants in this pathway are not known. However, one such mediator could be the tumor suppressor p53 based on the observation that Bcl-2 protein interferes with the p53-induced apoptosis without impairing the p53-induced G1/S arrest (Wang et al., 1993; Ryan et al., 1994; Selvakumaran et al., 1994). p53 was shown to induce apoptosis under a variety of conditions (reviewed in Sachs and Lotem, 1993; Hoffman and Liebermann, 1994; Liebermann et al., 1995) and in p53-deficient tumor cell lines upon expression of wild-type p53 (Yonish-Rouach et al., 1991; Shaw et al., 1992). It is not clear how p53 induces apoptosis. However, p53 was shown to negatively regulate the *bcl-2* gene through a *cis*-acting negative response element in its 5'-untranslated region (Miyashita et al., 1994b). Also, p53 was shown to increase the level of *bax* mRNA (Miyashita et al., 1994a; Selvakumaran et al., 1994). Bax is a Bcl-2-related gene product that was shown to accelerate PCD by forming heterodimers with Bcl-2, thus inactivating Bcl-2 (Oltavi et al., 1993). Recently, p53 was shown to increase *bax* mRNA levels by transactivating the *bax* promoter (Miyashita and Reed, 1995).

However, p53 is not the whole story of apoptosis, since p53 mediates apoptosis for only a particular set of signals (Lowe et al., 1993). Bcl-2 was also shown to inhibit p53-independent apoptosis (Sentman et al., 1991; Miyashita and

Reed, 1992 and 1993). By exploiting the yeast two-hybrid system (Fields and Song, 1989) to identify interacting proteins that mediate Bcl-2's functions, it was shown recently that Bcl-2 inhibits apoptosis through a novel protein called bcl-2 associated athanogene 1 (BAG-1) (Takayama et al., 1995).

Interestingly, BAG-1 also does not contain any protein motifs that would indicate a biochemical function. Furthermore, it showed no homology to Bcl-2 or Bcl-2-related proteins and any relationships between BAG-1 and p53 are not known at this time. However, gene transfer experiments showed that BAG-1 can prevent PCD (Takayama et al., 1995).

Latent and/or persistent infections are a part of the lifestyles of many viruses, as the ability to maintain a long-term relationship with the hosts is vital for their survival. A variety of viral mechanisms have evolved to prolong the life span of host cells one such mechanism is by exploiting the anti-apoptotic properties of Bcl-2. An example is Epstein-Barr virus (EBV), a DNA tumor virus which is ubiquitous and is associated with tumors of B cells and epithelial cells (Magrath, 1990; Shibata and Weiss, 1992). In B lymphocytes, the infection is non-productive or latent, whereas in epithelial cells, the infection is productive (Miller, 1990). The immediate early protein of EBV, BHRF-1, shows similarity to Bcl-2, Bcl-x and Bax (Bcl-2 homologs). Like bcl-2, BHRF-1 acts as a "cell survival gene" (Cleary et al., 1986). Furthermore, cells that are negative for EBV, but express

BHRF-1, showed strong resistance to apoptosis induced by growth factor deprivation (Henderson et al., 1993). Furthermore, the EBV latent EBNA-4 gene was shown to increase the expression of Bcl-2 (Silins and Sculley, 1995). The importance of these findings is that the viruses can harbor Bcl-2 homologs which are capable of inhibiting PCD. When taken together with the observations that Bcl-2 can block or delay apoptotic death of virus-infected cells and convert a lytic viral infection into a non-lytic persistent infection (Alnemri et al., 1992; Levine et al., 1993), these results suggest that virally-encoded Bcl-2 homologs contribute to viral latency or allow for persistent infections in the absence of cell lysis.

Analogous to EBV, the African swine fever virus is a DNA virus which remains persistent for a long time in peripheral blood monocyte/macrophages of infected pigs (Vinuela, 1987). The LMW5-HL protein of African swine fever virus also shows significant similarity to Bcl-2, suggesting a role for the viral protein in the suppression of apoptosis in virus-infected cells (Neilan et al., 1993). Another viral protein, the p35 baculovirus gene product, has the ability to block the host apoptosis response (Clem et al., 1991).

More recently, the adenovirus E1B 19K protein was also shown to exhibit a modest homology with Bcl-2 and can functionally act as a homolog of Bcl-2 (Chiou et al., 1994). E1B 19K was shown to inhibit the apoptosis that is induced by the E1A by inhibiting the Bak (for Bcl-2 homologous

antagonist/killer) which promotes the apoptosis (Farrow et al., 1995; Kiefer et al., 1995; Chittenden et al., 1995).

1.3 Eukaryotic Transcription

I have explained (section 1.2.3) several cellular trans-acting factors that bind to the cis-acting signals in the JCV enhancer. Before explaining the role of these factors in glial cell-specific expression of JCV, I would like to describe some general features of eukaryotic transcription.

1.3.1 Promoters and Enhancers

Most of the protein coding genes in eukaryotes are transcribed by RNA polymerase II (RNAP II). Each gene often contains two sets of elements; the basal promoter elements or minimal core promoter elements and the modulator promoter elements or enhancer sequences. The basal promoter elements usually determine the specificity of RNAP II and the direct basal level of transcription. On the other hand, modulator promoter elements enhance or reduce the basal levels of transcription. The basal promoter elements generally consist of a TATA motif, located approximately 30 nucleotides upstream of the transcription start site, and the initiator (Inr) element which specifies the start site (reviewed in Conaway and Conaway, 1993; Zawel and Reinberg, 1995).

Enhancers are usually recognized by proteins which are modular in nature, containing a DNA-binding domain and an

activation domain. Enhancers are of two types; inducible and temporal, or tissue-specific. Inducible enhancers respond to environmental cues, such as heat shock, heavy metals, viral infection, growth factors and hormones for the metallothionein, γ-interferon and c-fos genes (reviewed in Maniatis et al., 1987). Tissue-specific enhancers have a vital role in developmental and in tissue-specific gene expression. For example, the IgH gene enhancer expresses only in lymphoid cells (Maniatis et al., 1987; Mitchell and Tjian, 1989). Thus, the efficiency and tissue-specificity of gene expression is determined by the interplay between enhancer binding factors and trans-activators (reviewed in Tjian and Maniatis, 1994).

1.3.2 RNA polymerase II

The RNA polymerase II (RNAP II) initiates transcription of messenger RNA (mRNA) at promoters located 5' of the coding sequence of each gene. Human RNAP II is a complex molecule made up of 10 subunits with apparent molecular weights ranging from 10-240 kDa. The largest subunit has a unique carboxy terminal domain (CTD) with multiple serine, threonine, tyrosine and proline residues, suggesting that it is phosphorylated at serine, tyrosine and threonine residues. Furthermore, phosphorylated and unphosphorylated forms of RNAP II can be resolved on polyacrylamide gels (Woychike and Young, 1990). The role of the CTD in transcription initiation is still elusive. However, the genetic manipulation data suggest

that removal of the repeats in CTD is lethal to Drosophila, Yeast and murine cells (Allison et al., 1988; Bartolomei et al., 1988; Edwards et al., 1991). The role of the CTD in enhancer-driven transcription was recently demonstrated. Removal of most of the CTD abolished transactivation at most enhancers (Gerber et al., 1995) indicating that CTD is essential in mediating 'enhancer'-type transcriptional activation. Recently, it was suggested that the unphosphorylated form of the CTD is present in the preinitiation complex while the phosphorylated form is present in the elongation complex. Thus, the conversion of CTD from an unphosphorylated to a phosphorylated form may allow the transition from transcriptional initiation to elongation (Laybourn and Dahmus, 1990). Other proposed functions are RNAP II localization, DNA binding, removal of chromatin proteins from DNA and regulation of RNAP II activity (reviewed in Corden, 1990; Dahmus, 1994).

1.3.3. General transcription factors

The process of transcription initiation requires a concerted interaction between RNAP II and at least eight protein factors. These factors are termed general transcription factors (GTFs). A total of eight GTFs were identified in human cells and they are called TFIIA, B, E, F, H, I, J and D. Together with RNAP II, GTFs are necessary for the basal level of transcription. The first step in the

assembly of the initiation complex is the binding of TFIID to DNA. TFIID is the only component of basal initiation factors that contains DNA-binding capacity to the TATA box sequence. TFIID is a multimeric protein complex containing the TATA box binding protein (TBP) and TBP-associated factors (TAFs) (Pugh and Tjian, 1990). Human TFIID consists of TBP and at least 8 TAFs with molecular weights ranging from 30-250 kDa (Tanese et al., 1991). TBP requires no other factors for TATA element recognition and the TBP-TATA element complex subsequently incorporates the factors for basal transcription, but can not respond to transcriptional activators without TAFs (Pugh and Tjian, 1991; Hoey et al., 1993). In the assembly of the multimeric initiation complex the binding of TFIID to the TATA box appears to be the first step. The TFIID-DNA complex provides a recognition site for the association of other basal factors and RNAP II (Buratowski et al., 1989).

TFIIA promotes stable binding of TFIID to the core promoter. TFIIA was also shown to associate with TBP or the TFIID complex, even in the absence of DNA (Cortes et al., 1992). Several negative regulators of transcription were shown to displace TFIID from the promoter and others were shown to block TFIID's interaction with other GTFs (Auble and Hahn, 1993). TFIIA was shown to counteract these negative factors by its direct interaction with TBP (Reinberg et al., 1987).

TFIIB recognizes either the TFIID-DNA complex or the TFIID-DNA-TFIIA complex to form DB or DAB complexes,

respectively (Buratowski et al., 1989). Recently, TFIIB was shown to be critical for interaction between the initiation complex and upstream activators (Lin et al., 1991). It is also required for the recruitment of RNAP II. TFIIB also serves as a bridge between TFIID and RNAP II. TFIIB can interact with RNAP II in solution therefore, it is also possible that these two components could be preassociated upon entering the initiation complex (Conaway and Conaway, 1993).

TFIIF associates with RNAP II even in the absence of other factors and also it is an important basal factor, because it is absolutely essential for transcription initiation (Reinberg and Roeder, 1987). RNAP II recruitment is carried out by the small subunit of TFIIF, possibly via interaction with TFIIB (Flores et al., 1991; Ha et al., 1993). Besides acting as an initiation factor, TFIIF also affects elongation of RNAP II (Price et al., 1989).

Thus, TFIID, A, B, F and RNAP II form a minimal complex or preinitiation complex (PIC). For a complete initiation complex, TFIIE and H are necessary. However, under some circumstances the minimal complex can initiate transcription (Parvin and Sharp, 1993). TFIIE is necessary for further incorporation of TFIIH (Flores et al., 1992).

TFIIH is the last basal factor to enter into the complex. It is a multisubunit complex with many biochemical activities. The important activities are DNA helicase, DNA-dependent ATPase and CTD phosphorylation (Serizawa et al., 1993).

Finally, transcription initiation requires the hydrolysis of ATP or dATP (Sawadogo and Roeder, 1984; Goodrich and Tjian, 1994), and this process is termed the activation of the initiation complex. TFIIH may be the factor mediating activation, since it possesses ATPase activity.

In TATA-less promoters, TFII-I binds to the Inr elements and promotes TBP binding (Smale, 1994).

1.3.4 Sequence-specific transcription factors

Besides GTFs, additional transcription factors bind to specific DNA sequences in the upstream regulatory region. These are called transcriptional activators or repressors, depending whether they stimulate or repress transcription. A classical transcription factor has a specific DNA-binding domain, a hinge or multimerization domain for the formation of homodimers or heterodimers and an activation domain. DNA-binding domains are classified as; the helix-turn-helix (HTH) domain, the homeodomain, zinc finger segments, the steroid receptor binding domain, the leucine zipper domain, the helix-loop-helix domains and the β-sheet domain (reviewed in Pabo and Sauer, 1992).

Several other activation domains are classified as acidic, glutamine rich and proline rich activation domains (Mitchell and Tjian, 1989). After interaction of sequence-specific transcription factors with enhancers, they contact the basal transcription machinery to directly or indirectly

stimulate the rate of transcription (reviewed in Ptashne and Gann, 1990). Not all activation domains of a given class interact with the same target. Hence, there may be several distinct types of activators acting on the same activation domain. Interestingly, the important amino acid residues for activation are not necessarily the abundant glutamines, acidics or prolines. The hydrophobic residues interspersed among these abundant residues are important for activation (Gill et al., 1994).

1.3.5 Mechanism of transcriptional activation by transcription factors

Gene expression in eukaryotes requires sequence-specific transcription factors that bind to sets of cis-acting elements specific to each gene (Mitchell and Tjian, 1989; Ptashne, 1988). Trans-acting factors may be ubiquitous, tissue-specific or stage-specific and they can interact synergistically with each other, thus forming a unique nucleoprotein complex (Sawadogo and Sentenac, 1990). Several steps in the assembly of the initiation complex are potential targets for trans-activators. These trans-activators may facilitate TBP binding to TATA box, which in turn accelerates the recognition of the promoter by the GTFs. Alternatively, they may stabilize the binding of TBP to weak TATA boxes. Several mechanisms have been postulated for transcriptional activation. The greater details of the mechanisms are discussed in the following

sections.

1.3.5.1 Interaction with GTFs

TFIID binding to the TATA box is the first step in the assembly of the PIC. TFIID was thought to be the most likely target for activators. Yeast Gal4 (Horikoshi et al., 1988b), mammalian activating transcription factor (ATF) (Horikoshi et al., 1988a) and upstream stimulatory factor (USF) (Sawadogo and Roeder, 1985) were shown to target TFIID and this interaction was shown to activate transcription. Other activators, like herpes simplex virus VP16 (Stringer et al., 1990), papilloma virus E2 protein (Dostani et al., 1991) and Epstein-Barr virus Zta protein (Lieberman and Berk, 1991), enhance transcription by interacting and stabilizing the TFIID complex at the promoter. The pseudorabies virus immediate early protein (IE) stimulates transcription by accelerating the recruitment of TFIID to the TATA box (Abmayr et al., 1988). The tumor suppressor p53 was shown to interact with TBP or partially purified TFIID and activated transcription by the formation of a more stable TFIID-DNA complex (Chen et al., 1993; Traunt et al., 1993). The other activator, PU.1, was shown to interact with TBP directly (Schuetze et al., 1992). In addition to TFIID, other GTFs were also likely to be the targets for activators. The VP16 activation domain was shown to stimulate transcription by recruiting TFIIB to the PIC (Lin and Green, 1991). CREB was also shown to interact directly

with TFIIB and TFIID. Thus, CREB may promote basal transcription initiation through interaction with TFIID and by recruiting TFIIB to the promoter (Xing et al., 1995). Cellular transcription factor LSF was also shown to accelerate the association of TFIIB with TFIID bound to the TATA box (Sundseth and Hansen, 1992).

1.3.5.2 Interaction with TAFs/coactivators

The interaction between trans-activators and GTFs may contribute to, but is not sufficient for the process of activation because studies with purified TBP exhibited basal, but not activated, levels of transcription, even in the presence of sequence-specific transcription factors. Interestingly, the addition of partially purified TFIID reconstituted the activated levels of transcription. This suggests that TAFs are important for activated transcription (Pugh and Tjian, 1990). A subset of TAFs are also called coactivators, since they interact with activators and form a bridge between TBP and activators. Subsequent identification of TAFs lead to the coactivator hypothesis (Dynlacht et al., 1991; reviewed in Tanese and Tjian, 1993). Some TAFs are tightly and stoichometrically associated with TBP and some are transiently associated with TBP (Drapkin et al., 1993). For example, an activity termed upstream stimulatory activity (USA) is required for activation by SP1, USF and a coactivator required for trans-activation by serum response factor (SRF)

can be separated from TFIID in chromatographic columns (Meisternest et al., 1991; Zhu and Prywes, 1992).

Subsequently, TAFs were isolated from TFIID and addition of these TAFs to the recombinant TBP stimulated the rate of transcription in the presence of transcription activators (Dynlacht et al., 1991). Thus, activators may interact with one or more of these TAFs directly to regulate the activity of TBP.

As of this date, 8 TAFs have been isolated which are important for activator-mediated transcription for RNAP II. Only a few have been cloned, the human $TAF_{II}250$ and Drosophila $TAF_{II}110$, 80, 40 and 150 (Dynlacht et al., 1993; Goodrich et al., 1993; Hisatake et al., 1993; Hoey et al., 1993; Ruppert et al., 1993; Verrijzer et al., 1994). Human $TAF_{II}250$ was shown to be involved in cell cycle progression (Wang and Tjian, 1994). Drosophila $TAF_{II}110$ interacts with $TAF_{II}250$ and TAF_{II} 30α and, thus, is recruited to TBP which is bound to the TATA box (Kokubo et al., 1993). It also interacts with TFIIA (Yokomori et al., 1993). Drosophila $TAF_{II}250$ and 110 serve as coactivators for SP1 by bridging SP1 with TBP to stimulate the rate of transcription (Weinzierl et al., 1993). The acidic activator, VP16, was shown to interact with $dTAF_{II}40$ to activate transcription (Goodrich et al., 1993). CREB was also shown to interact with $dTAF_{II}40$ through the regions, similar to that of SP1 (Gill et al., 1994). The acidic transcriptional activation domain of p53 was shown to interact with $hTAF_{II}40$

and 60 for transcriptional activation (Thut et al., 1995). The $dTAF_{II}150$ was shown to interact with the DNA sequences overlapping the transcription start site. Thus, together with TBP, it is responsible for TFIID interaction with the core promoter (Verrijzer et al., 1994). This suggests that different classes of activators interact with distinct TAFs. In addition to the interaction of TAFs with activators, they may serve as a bridge between TFIID and other basal transcription factors (Choy and Green, 1993). More recently, TAFs were shown to play a critical role in promoter selectivity and transcriptional regulation through direct contacts with core promoter elements (Verrijzer et al., 1995). Thus, different activators interact with different TAFs of the multisubunit TFIID complex, contributing to the specificity of gene regulation (Ruppert et al., 1993).

The role of coactivators in tissue-specific gene expression in eukaryotes is also well documented. For example, the lymphoid cell-specific coactivator was shown to be important for IgH gene transcription (Luo et al., 1992). Another case is hepatocyte nuclear factor-1 (HNF-1), which activates liver specific-gene expression as a dimer. This dimerizatic n was shown to be mediated by a liver specific-coactivator (Mendel et al., 1991).

In general, activators, TAFs and GTFs may participate at different steps and in specific orders during the dynamic process of initiation of transcription. The existence of

several TAFs, their tissue and developmental distribution and their putative tissue-specificity also imply that different classes of activators interact directly with distinct TAFs.

1.3.5.3 Interaction with adaptors and accessory proteins

Besides TAFs/coactivators, trans-activators interact with other proteins, such as adaptors and accessory proteins. An adaptor can bind to a DNA-binding protein and mediate transcriptional activation through its activating region. Accessory proteins help in DNA-binding, complex assembly or activation (Stanway, 1991).

One example of an adaptor is the herpes virus VP16 protein. VP16 binds to the DNA along with Oct-1 and two other cellular proteins, C1 and C2 (McKnight et al., 1987; Kristie et al., 1989). Besides activating transcription through its activation domain via a coactivator, VP16 also allows Oct-1 to activate transcription by adapting Oct-1 to the general transcription machinery. Unlike the classical adaptor, as defined above, VP16 also makes direct DNA contact (Kristie et al., 1990). Two functional studies with the VP16 activation region have led to a novel hypothesis called "squelching", for inhibition of transcription due to the overexpression of an activating region that competes with a functional activator for the binding sites of targets (reviewed in Ptashne, 1988; Gill and Ptashne, 1988). The VP16 activation domain was fused to the Gal4 DNA-binding domain. The chimeric Gal4-VP16

construct was shown to inhibit the activated, but not the basal, level of transcription from the dA:dT upstream activating sequence (UAS)-promoter (Berger et al., 1990). The same study also demonstrated that inhibition of activated transcription does not depend on DNA-binding. This suggested that VP16 binds to a targeted factor in the transcriptional apparatus. The specificity of this effect for activated transcription suggests that such a target is necessary for activated, but not basal, transcription. One hypothesis is that such a target is an adaptor that connects trans-activators to the basal apparatus, by titrating out the adaptor. Thus, Gal4-VP16 prevents the activating effect of the dA:dT activator. Similar results were obtained in another study using Gal4-VP16 and the dA:dT UAS-promoter. This study (Kelleher et al., 1990) ruled out the possibility of a GTF acting as an adaptor, as speculated by the above study (Berger et al., 1990).

Similarly, adenovirus E1A was shown to require an adaptor for transactivation, based on squelching experiments (Martin et al., 1990). Overexpression of the E1A activating region inhibited E1A activation in vivo and in vitro (Martin et al., 1990). Subsequently, cellular transcription factors, like CTF/NF-1 and SRF, were shown to require a coactivator/ adaptor/cofactor for activation. In all the cases, overexpression of the activating regions lead to the squelching or inhibition of the activated level of

transcription (Martinez et al., 1991; Prywes and Zhu, 1992).

Such squelching experiments provide much of the knowledge of adaptors. Though these adaptors are normally present in the cell, they do not squelch transcription. It is possible that, conformational changes or cooperativity may be required for such an activation.

In general, transcriptional adaptors, accessory proteins and coactivators provide an explanation for how wide varieties of transactivating regions can interact with a limited set of basal factors. These factors also represent additional steps in the process of transcriptional activation that are potentially subject to regulation in the cell.

1.3.6 Transcriptional regulation of tissue-specific gene expression

Though cell-specific gene expression is regulated at different levels, transcriptional regulation plays a crucial role. The upstream cis-acting elements and their cognate transcriptional factors involved in tissue-specific gene expression are well documented in several cases. For example, gene expression was shown to be controlled mainly at the transcriptional level in the muscle, pituitary, erythroid, immune system and brain. Among these different organs, the brain is a complex one containing a heterogeneous population of cells. Hence, it is difficult to study brain-specific gene expression. The prototype human JCV expresses and replicates

only in the brain. Thus, JCV serves as a good model for the study of brain-specific gene expression (Major et al., 1992). JCV enhancer sequences share some similarities with the regulatory regions of myelin basic protein (MBP), glial fibrillary acidic protein (GFAP) and proteolipid protein (PLP). JCV and all these brain-specific genes have NF-1 and AP-1 binding sites in their regulatory regions. Also, the importance of NF-1 binding sites was well documented for all of these genes (Aoyoma et al., 1990; Besnard et al., 1991; Nave and Lemke, 1991).

1.4 Glial cell-specific expression of JCV

The promoter-enhancer sequences of JCV were identified, based on sequence comparison with other polyomaviruses. A unique feature of JCV is that the TATA box is duplicated, unlike that of SV40 (Frisque et al., 1984). It was thought that the 2 JCV TATA boxes would control early and late transcription of the virus.

The first report to demonstrate that the 98 bp tandem repeats act as promoter-enhancer signals was from Kenney et al (1984). They fused JCV promoter-enhancer signals to the CAT reporter gene in two orientations and assayed activity in PHFG, HeLa, HEK and CV-1 cells. The highest CAT activity was observed in PHFG. In contrast, SV40 regulated CAT activity was found in all cells. These results suggest that, unlike other polyomaviruses, the JCV promoter-enhancer is strictly glial

cell-specific.

Chimeric virus studies also demonstrated the importance of the JCV regulatory region in glial cell-specific expression. When the JCV regulatory region was replaced with that of SV40 or BKV, the hybrid constructs were not viable in PHFG cells. Whereas, the hybrid SV40 and BK viruses containing the JCV regulatory region could control the synthesis of SV40 or BK T-antigens in glial cells. This suggests that SV40 or BK T-antigens can interact with the JCV regulatory region, but that the reverse is not true. Furthermore, this implies that JCV T-antigen plays a significant role in glial cell-specific expression of JCV (Chuke et al., 1986).

In Section 1.2.4, several trans-acting factors interacting with the JCV regulatory region were introduced. Though several factors have been identified by so-called in vitro "promoter-bashing" experiments, virtually no studies indicate which interaction is essential in vivo. Added to this, a recent report demonstrated that the JCV minimal core promoter element containing only the TATA box and an 8 bp poly(T) region, without the aid of upstream cis-elements, was able to support glial cell-specific transcription (Krebs et al., 1995). Thus, the role of these transcription factors in glial cell-specific expression is questionable.

Nevertheless, I would like to discuss the importance of the interaction of some of these transcription factors in the glial cell-specific expression of JCV. In this laboratory, we

used P19 embryonal carcinoma (EC) cells as a model system to study the glial cell-specific expression of JCV. EC cells are a good model system to study development. They are the malignant stem cells of a teratocarcinoma and are pluripotent in nature, similar to cells of the inner cell mass of preimplantation mouse embryos (Martin, 1980). When EC cells were subjected to different chemical treatments they yielded different cell types with characteristic features of the cells of three of the germ layers (Martin, 1980). This suggested that the mechanism of differentiation of EC cells is similar to that of normal embryonic cells (McBurney et al., 1988). Furthermore the EC system allows experiments requiring different cell phenotypes of an identical genotype.

Addition of retinoic acid (RA) to EC cells in monolayers or aggregated cells gave rise to different kinds of differentiated cells. Furthermore, the concentration of RA had an effect on this process. For example, when 10^{-6} M RA was added to monolayers of EC cells, the result was a heterogeneous population which were endoderm-like, mesoderm-like and a few were neuron-like cells (Jones-Villeneuve et al., 1983). When added at $3x10^{-7}$ M to aggregated cells, RA treatment yielded a heterogeneous population of neuronal cells consisting of neurons, astroglia, microglia and fibroblast-like cells (McBurney, 1993). EC cells can be differentiated into skeletal and cardiac muscle cells by treating the undifferentiated cells (UD) with dimethyl sulfoxide (DMSO)

(McBurney, 1993).

A previous study from our laboratory demonstrated that JCV was expressed in P19 glial cells, but not in P19 muscle or UD cells (Nakshatri et al., 1990a). Thus, these cells paved the way for further studies of the glial cell-specific expression of JCV. Although the UD EC cells can be differentiated into different cell types from an identical genotype, the P19 RA-differentiated cells give a heterogenous population of neuronal cells. This is the disadvantage of the EC system. Nevertheless, I also employed these cells in my study, since previous studies observed efficient expression of JCV in P19 glial cells (Nakshatri et al., 1990a; Kumar et al., 1993). (**Note:** Recently, I have demonstrated that 70% of the P19 RA-differentiated cells are glial cells which are positive for GFAP, a marker specific for glial cells. Therefore, I have referred to RA-differentiated cells as P19 glial cells. Similarly, DMSO-differentiated cells are referred to as P19 muscle cells, throughout the text).

Previously, DNase I footprinting with nuclear extracts from UD, P19 glial and P19 muscle cells were used to detect the proteins interacting with the JCV regulatory region. Only the P19 glial cell nuclear extracts protected three regions. Two protected regions were observed within the 98 bp repeats and one was towards the late side and outside the repeats. All these regions contained sequences homologous to NF-1 binding motifs. The regions within the repeats were named regions II

and III and the region outside the repeat was named region I (Nakshatri et al., 1990a).

Other studies using in vitro binding assays demonstrated binding to the NF-1 sites in the JCV regulatory region (Khalili et al., 1988). The same study showed that JCV nt 62-160 was bound by protein(s) from human fetal glial cells in a tissue-specific manner. The exact sequence was identified as an NF-1 motif by DNase I footprinting with mouse brain nuclear extracts (Tamura et al., 1988). The same study also demonstrated the importance of the NF-1 motif as a cis-acting element for glial cell-specific expression using in vitro transcription techniques. Amemiya et al (1989) have shown that three regions, nt 33-58, site A; nt 131-156, site B; and nt 209-230, site C, can be protected in the JCV regulatory region in DNase I footprinting experiments with extracts from glial cells. The protected regions were shown to be homologous to NF-1 motifs and the binding that they showed by gel shift assay could be competed with an oligonucleotide homologous to the NF-1 site of the adenovirus DNA origin of replication. They concluded that an NF-1 or an NF-1-like factor was interacting with the JCV regulatory region. However, there were no prior reports to clearly demonstrate the importance of these NF-1 motifs (Kumar et al., 1993).

NF-1 was first identified as a factor from HeLa cells required for adenoviral DNA replication (Nagata et al., 1982) and was shown to interact with adenovirus DNA origin of

replication (Nagata et al., 1983; Rawlins et al., 1984). Subsequent work has demonstrated the importance of NF-1 in transcription of the adenovirus major late promoter (Gronostajski et al., 1988). The same is true for many cellular genes, like those encoding human α1-globin (Jones et al., 1987), mouse albumin (Lichstener et al., 1987), mouse α2(I) collagen (Rossi et al., 1988), chicken lysozyme (Borgmeyer et al., 1984), human IgM (Hennighausen et al., 1985), c-myc (Siebenlist et al., 1984), adipocyte gene (Graves et al., 1991), ovalbumin (Bradshaw et al., 1988) and several other proteins expressed in the CNS (Amemiya et al., 1992). In particular, the importance of NF-1 in viral gene expression also emerged from the finding that this protein binds and activates a large fraction of the viral regulatory elements. For example, NF-1 activates the cytomegalovirus (CMV) promoter (Hennighausen and Fleckenstein, 1986), human papilloma virus type 16 (HPV16) and 18 (HPV18) enhancers (Nakshatri et al., 1990b; Chong et al., 1990), human BKV polyoma virus promoter (Nowock et al., 1985), the long terminal repeat (LTR) of feline leukaemia virus (Plumb et al., 1991) and the hepatitis B virus enhancer (Ben-Levy et al., 1989). Interestingly, the MMTV-LTR was used to show a unique cooperation between NF-1 and the glucocorticoid receptor (Archer et al., 1992).

The minimum number of nucleotides required for NF-1 binding is 15-16, with a two-fold axis of symmetry, separated by a 6 nucleotide spacer (TGGA/CNNNNNNGCCAA) (de Vries et al.,

1985; Leegwater et al., 1985). Though the first 3 nucleotides in the first motif and the spacer region are sufficient for NF-1 binding, the presence of A/C at the 4th position in the 5' half site increases the affinity to NF-1. The NF-1 sequences found in the JCV regulatory region contained the optimal fourth nucleotide and spacer (Amemiya et al., 1989; Nakshatri et al., 1990a). Another study showed that CCAAT transcription factor (CTF) can interact with part of the NF-1 consensus sequence. Subsequently, it was demonstrated that CTF and NF-1 are related but are distinct from each other (Chodosh et al., 1988; Raymondjean et al., 1988). Further, isolation of cDNAs for NF-1 proteins from different tissues confirmed this finding and showed that these cDNAs had homology at the region necessary for DNA binding (Paonessa et al., 1988; Santoro et al., 1988; Rupp et al., 1990). Some of these cDNAs were shown to arise from alternative splicing (Mermod et al., 1989). Others were coded by different genes (Gil et al., 1988; Paonessa et al., 1988). Since NF-1 mediates transcription and replication, it is also possible that both processes could be functionally coupled by the binding of CTF/NF-1 factors to common DNA sequences (de Pamphilis, 1988).

NF-1 is a large family of proteins. Some NF-1 proteins are tissue-specific in regulating the expression of genes. For example, an epithelial specific NF-1 protein interacts with an NF-1 motif in the HPV 16 enhancer and regulates the epithelial-specific expression of the virus (Apt et al.,

1993). Similarly, HPV 11 contains multiple NF-1 motifs and expresses efficiently in P19 glial cells (Kasinadhuni, 1994). Since JCV also expresses efficiently in these cells, it is not clear whether the same factor(s) is involved in the expression. Interestingly, BKV also contains NF-1 motifs in its regulatory region but expresses efficiently in both P19 glial and non-glial HeLa cells, unlike JCV (Nakshatri et al., 1991). This suggests that different NF-1s in these two cell types regulate the differential expression of these two related viruses. The glial cell-specific NF-1 is essential for the restricted glial cell-specificity of JCV. Furthermore, nuclear extracts from brain cells completely protected the NF-1 motifs in the JCV regulatory region, whereas the HeLa cell extracts protect partially (Amemiya et al., 1989). This suggests that the NF-1 in HeLa cells may be different from the NF-1 of brain cells. Interestingly, the protected regions can be competed with oligonucleotides homologous to NF-1 motifs but not to CTF motifs. This further indicated that not all the NF-1 family of proteins interact with the JCV NF-1 motifs (Amemiya et al., 1989).

To conclusively test the importance of these NF-1 sites, these motifs were destroyed by site-directed mutagenesis and assayed functionally (Kumar et al., 1993). Mutation of one of the NF-1 motifs in 98 bp repeats resulted in 3.5- to 4-fold reduction and mutation of both motifs resulted in a 7-fold reduction in the activity of the JCV_E. Mutation of the NF-1

motif in region I had no effect. Thus, the results showing that NF-1 motifs present in the repeats, but not the one outside the repeats are important for JCV expression is a novel study. The same study also confirmed this finding by in vitro transcription assays (Kumar et al., 1993). Studies from our lab and from those of others suggested that protein(s) binding to the NF-1 motifs are important for JCV transcription (Ahmed et al., 1990a; Amemiya et al., 1989, 1992; Kumar et al., 1993). NF-1 motifs were also shown to be essential for JCV DNA replication. Mutations in NF-1 motifs completely abolished DNA replication (Sock et al., 1991).

Like JCV_E, the JCV_L was also glial cell-specific. Only a low level of JCV_L activity could be detected in glial cells, but, in the presence of JCV T-antigen, a 9- to 12- fold activation in glial cells and a 4-8 fold activation in nonglial cells was observed. This suggested that T-antigen acts as a transactivator (Lashgari et al., 1989). However, the mechanism of transactivation was not clear. Work from our lab has shown that the integrity of the NF-1 motifs in the 98 bp repeats is important. Furthermore, T-antigen was shown to facilitate an increased binding of NF-1 to its JCV NF-1 site (Kumar, 1994).

Since the JCV NF-1 motifs played an essential role in glial cell-specific expression of JCV, further studies were done in this laboratory to clone the protein(s) that interacts with the NF-1 motifs. To this end, a cDNA was detected and

cloned from a P19 glial cell expression library, using an NF-1 region II/III probe using Southwestern blot assays. The cDNA was preliminarily characterized and was suggested to stimulate the expression of JCV_E and JCV_L to an equal extent in the HeLa cell system (Kumar, 1994). At the same time, work from another laboratory isolated a clone from a human brain cDNA library, using a probe that partially overlaps the region II/III NF-1 motif (Kerr and Khalili, 1991). This cDNA was shown to code for a 45 kDa protein that stimulated the expression of JCV_L, but not JCV_E. Their cDNA did not reveal any homology to NF-1 or NF-1-related genes.

Attempts were also made to purify the protein(s) interacting with NF-1 motifs. A 45 kDa protein was purified from calf brain using a partially overlapping sequence corresponding to the NF-1 region II/III. This protein was shown to transactivate JCV_E in *in vitro* transcription assays (Ahmed *et al.*, 1990a). Although a cDNA and a protein were both isolated in Khalili's lab, using the same NF-1 region II/III probe, the results were contradictory. Though the expression of the cDNA isolated in our lab transactivated both JCV promoters in HeLa cells, it was not clear whether this activation required the integrity of the region II/III NF-1 sites.

Different factors were involved in the activation of JCV_E and JCV_L. The cDNA was shown to transactivate both JCV_E and JCV_L in non-glial cells, further confirming that the protein

encoded by the cDNA is important, but may not be solely responsible for the glial cell-specific expression of JCV. However, neither the specificity of binding of the protein encoded by the cDNA nor its structure-function studies were determined. The fact that the protein encoded by a cDNA stimulated the transcription of both JCV promoters raises an interesting possibility of the existence of alternatively spliced products of the putative factor. The basis for this assumption is an earlier demonstration that different members of the NF-1 family of proteins arise from alternative splicing (Santoro et al., 1988; Mermod et al., 1989). From the previous studies in our lab, it was not clear whether our cDNA codes for NF-1 or not. Hence, to address the above-mentioned questions and to characterize the cDNA, I initiated my work with the following hypothesis and specific aims.

1.5 Hypothesis and specific aims

1.5.1 Hypothesis

JCV is a neurotropic virus in vivo and in vitro and the regulation of gene expression is central to this neurotropism. Our lab had previously found two NF-1 sites in the JCV regulatory region that are essential for JCV expression. The cDNA clone isolated by Southwesternblot analysis in filter binding assays was used to demonstrate the importance of a protein encoded by the cDNA in the glial cell-specific expression of JCV. However, the characterization of the cDNA

was not carried out in detail. The structural features of the protein encoded by cDNA can address, to a certain extent, the above mentioned questions. Therefore my specific aims are:

1.5.2 Specific aims

The purpose of the present study was:

1. To sequence the cDNA completely.
2. To characterize the protein encoded by this cDNA using *in vitro* and *in vivo* assays.
3. To delineate any possible DNA binding and transactivation domains of the factor.
4. To study the other possible functions of the cDNA in JCV expression.

These studies were pursued to provide insight into the detailed molecular mechanism for the glial cell-specific expression of JCV.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Restriction enzymes, T4 DNA ligase, DNase I/DNA polymerase I were purchased from Bethesda Research Laboratories (GIBCO-BRL). Calf intestinal alkaline phosphatase was from Boehringer Mannheim. Reverse transcriptase (RT) was from Life Sciences. Thrombin was purchased from Novagen. Radiochemicals, like [^{14}C]chloramphenicol (ICN Biomedicals), [α^{32}P]dCTP, [γ^{32}P]dATP, [^{35}S]dATP, [^{35}S]methionine and [^{3}H]leucine were purchased from Amersham.

Alpha modified Eagle's medium (αMEM) was from ICN Biomedicals. Phosphate buffered saline (PBS) was from Flow Laboratories. Dulbecco's modified Eagle medium (DMEM), fetal calf serum and trypsin-EDTA were purchased from GIBCO-BRL. All trans-retinoic acid (RA), acetyl coenzyme A (coA), α-amanitin, bovine serum albumin fraction V (BSA), Nonidet P-40 (NP-40), dithiothreitol (DTT), sodium dodecyl sulfate (SDS) and protease inhibitors like antipain, pepstatin, aprotinin, phenylmethylsulfonylfluoride (PMSF) were from Sigma.

Synthetic oligonucleotides were from General Synthesis and Diagnostics (GSD), Toronto. Poly(dI-dC).poly(dI-dC) was purchased from Pharmacia. The XbaI translation termination linker ligated at the 3'-end of the mutant cDNAs was

commercially obtained from New England Biolabs. Nitrocellulose filters were purchased from Schleicher and Schuell. The total RNA isolation system, TNT *in vitro* translation system, primer extension system and RNasin (ribonuclease inhibitor) were from Promega. Sequenase Version 2.0 kit was from United States Biochemical (USB) for DNA sequencing. Bio-Rad protein assay kit was used to determine the protein concentration in whole cell extracts. GST-fusion protein purification system, pGEX-4T-1 vector and E.coli BL21 cells were from Pharmacia. The monoclonal antibodies to BAG-1 and Bcl-2 were from Santa Cruz Biotechnology and Pharmingen, respectively. The anti-rabbit IgG FITC conjugate was from Sigma. Dimethyl sulfoxide (DMSO) was from Baker. Triton X-100 was from New England Nuclear.

The P19 embryonal carcinoma and HeLa cells were maintained in this laboratory. U87 MG human glioblastoma cells were purchased from American Type Culture Collection (ATCC). Eukaryotic expression vector pRc/CMV was purchased from Invitrogen. Thin layer chromatography (TLC) plates and X-ray film were purchased from Kodak.

2.2 Methods

2.2.1 cDNA sequence analysis

The *bag-2* cDNA in pBluescriptIIKS$^+$ (pBSIIKS$^+$) was used for double-strand DNA sequencing. DNA sequencing was performed using Sequenase Version 2.0 kit, using conditions that are recommended by the supplier. Internal sense and antisense

strand primers were used to obtain the complete cDNA sequence and for verification. Where bands on a sequencing gel were ambiguous or compressed, reaction mixtures containing dITP, instead of dGTP were included. The sequence analysis was done through NCBI BLAST E-mail server (blast@ncbi.nlm.nih.gov) with the sequences in GenBank (release 89), GenBank update and non-redundant protein data bases. Further structural analysis of the cDNA was done with the appropriate programs in Pedro Biological Research tools available on world wide web (the URL ishttp://www.public.iastate.edu/~pedro/research_tools.html.) and the programs available in the P/C Gene program.

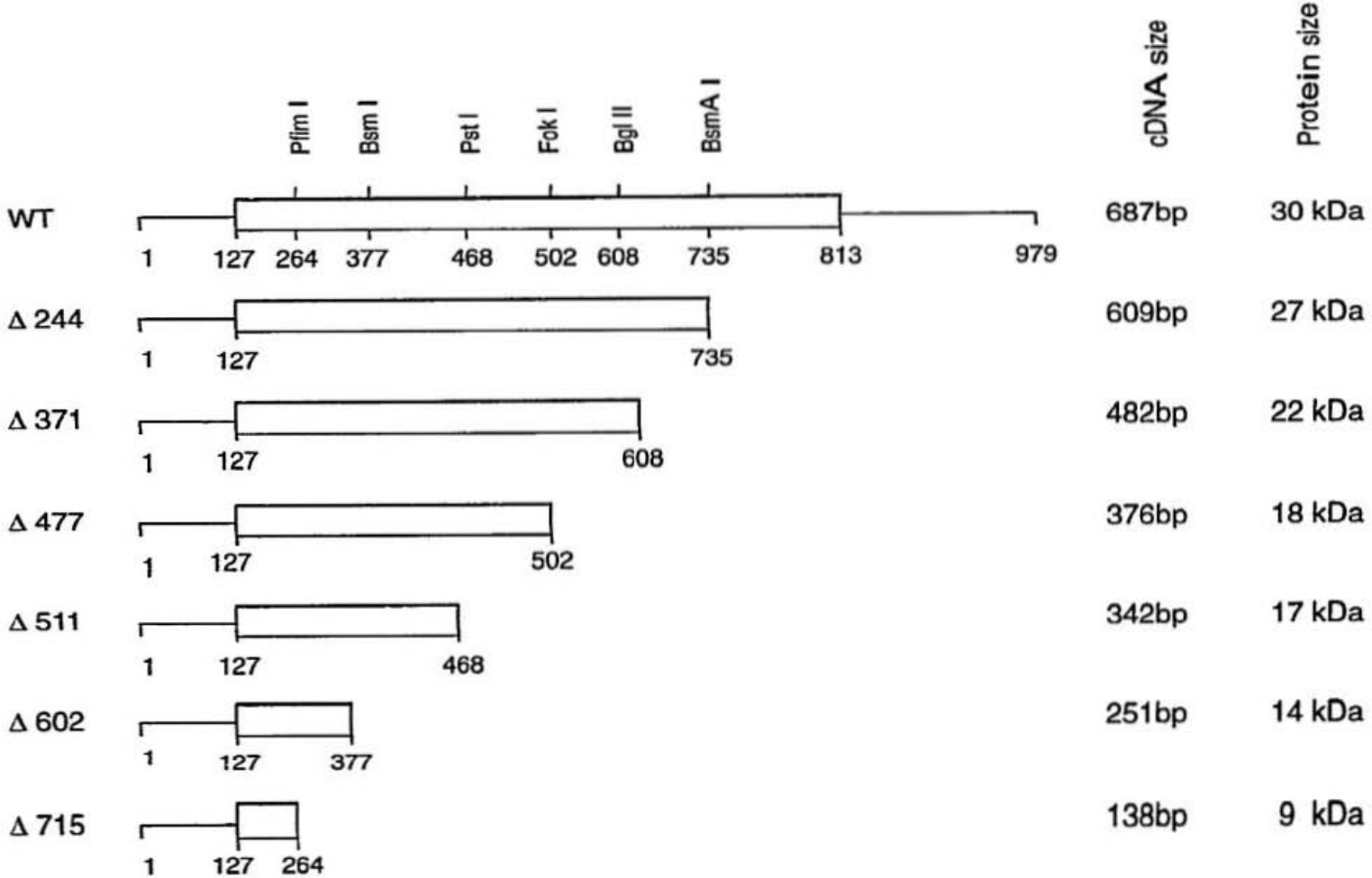
2.2.2 Deletion analysis of bag-2 cDNA

The cDNA was truncated at definite points with restriction enzymes. The truncated cDNAs were verified for sizes in polyacrylamide gels and recloned into either pRc/CMV or pBSIIKS$^+$. For cloning into pRc/CMV, translation termination linker having a termination codon in all of the three ORFs was ligated at the 3'-end to the mutants. The truncated cDNAs were numbered for the sizes of deletions from the 3'-end. Plasmids Δ244, Δ371, Δ477, Δ511, Δ602, Δ715 had deletions of 78, 205, 311, 345, 430 and 549 nt, respectively. Fig. 2.1 shows the sizes of deletions, their encoded protein sizes and restriction enzyme sites used to generate them.

2.2.3 Cell culture, differentiation of EC cells and

Fig. 2.1. Deletion analysis of *bag-2* cDNA.

The cDNA was truncated at definite points with the restriction enzymes, as indicated in the figure. The numbers on right indicate the number of bases deleted and the corresponding size of the protein coded by that truncated cDNA. The lines represent the untranslated sequences and the boxes represent the ORF of the cDNA. The figure is not drawn to scale.


Pflm I
Bsm I
Pst I
Fok I
Bgl II
BsmA I
cDNA size
Protein size
WT
1 127 264 377 468 502 608 735 813 979
687bp
30 kDa
Δ 244
1 127 735
609bp
27 kDa
Δ 371
1 127 608
482bp
22 kDa
Δ 477
1 127 502
376bp
18 kDa
Δ 511
1 127 468
342bp
17 kDa
Δ 602
1 127 377
251bp
14 kDa
Δ 715
1 127 264
138bp
9 kDa

transfection

The EC cells were cultured in αMEM supplemented with 10% heat-inactivated calf serum. These cells were differentiated according to the method described by Rudnicki and McBurney (1987). Briefly, confluent monolayers of cells were trypsinized and suspended in αMEM containing 300 nM all-trans retinoic acid (RA) or 1% DMSO and plated and incubated for 48 hrs. Bacterial culture plates were used to prevent attachment to the surface and incubated for 48 hrs. Another 48 hr incubation was continued after the addition of fresh media containing RA or DMSO. Then, the cells were washed with PBS, trypsinized and plated onto tissue culture plates containing medium without RA or DMSO.

Eight to ten hrs after plating, cells were transfected with the indicated amounts of test plasmids. In all cases the amounts of DNA was adjusted to 20 μg with pUC19 or pCMV. The DNA was transfected according to the calcium phosphate precipitation protocol of Graham and van der Eb (1973). The indicated amounts of DNA was mixed with 500 μL of 0.25 M $CaCl_2$ and 500 μL of 2X bis buffered saline (BBS) and incubated at room temperature (RT) for 13 min. The DNA mixture was added to cells in drops and the plates were incubated for 18–20 hrs at 37^0 C and in 3% CO_2. Then, the cells were washed two times with PBS and incubated at 37^0 C with 5% CO_2 with fresh medium for 48 hrs before harvesting.

HeLa and U87 MG cells were cultured in DMEM supplemented

with 10% heat-inactivated calf serum, 50 U of penicillin and 50 µg of streptomycin per mL of medium. Confluent monolayers were subcultured at a 1:5 ratio. Cells were transfected by the calcium phosphate precipitation method 8 hrs after plating as described above, with the exception of glycerol shock, at 4 hrs post transfection.

2.2.4 Chloramphenicol acetyl transferase (CAT) assay

Cells were harvested 48 hrs post transfection, as described (Gorman et al., 1982). Cells were washed with PBS, scraped with rubber policemen and collected into Eppendorf tubes. Following pelletization by centrifugation, the cell pellet was resuspended in 0.25 M Tris-HCl pH 7.8. Cells were disrupted by three cycles of freezing and thawing in liquid nitrogen and in a 37^0 C water bath for 5 min. The cell debris was pelleted by microcentrifugation and the resultant supernatant was aspirated and stored at -20^0 C.

Ten µL of the supernatant was incubated in a reaction mixture containing 14 µL of 1 M Tris-HCl pH 7.8, 4 µL of 4 mM acetyl CoA, 1 µL water and 0.1 µCi of [^{14}C]chloramphenicol at 37^0 C for 1 hr. Following incubation, the [^{14}C]chloramphenicol acetylated products were extracted in 500 µL ethyl acetate and dried in vacuum. The dried samples were resuspended in 15 µL of ethyl acetate, spotted on TLC plates and subjected to ascending TLC in a jar containing chloroform:methanol (95:5) to separate the acetylated and non-acetylated forms of

[^{14}C]chloramphenicol. Dried TLC plates were subjected to autoradiography. Percentage acetylation was determined by liquid scintillation counting. The pRSV-β-Gal plasmid was used as an internal control for transfection efficiency. The β-galactosidase assay was performed, as described (Kumar, 1994).

2.2.5 Plasmids

The recombinant plasmids were constructed according to the standard procedures described in Sambrook et al., (1989). Wild type JCV_E and JCV_L CAT constructs, containing the JCV regulatory region, with the difference being that they were in reverse orientation were made by Nakshatri et al., (1990a). The II_{10} CAT containing mutations in NF-1 motifs in regions II and III and DM_{10} CAT containing mutations in NF-1 motifs in regions I, II and III were described earlier (Kumar et al., 1993). The plasmid $pRII_ECAT$ containing only a single 98 bp repeat in an early orientation was constructed previously (Kumar et al., 1993). The plasmid pUC19-JC containing the JCV regulatory region in pUC19 at XbaI site was made by Kumar et al., (1993).The pCMV-BAG-2 plasmid was constructed by inserting the cDNA, isolated using the JCV NF-1 region II/III probe from P19 glial cell cDNA library, at the HindIII and NotI sites of pRc/CMV from Invitrogen (Kumar, 1994). The plasmid, pBSIIKS$^+$-BAG-2, was constructed by inserting the cDNA at the SalI and NotI sites of the pBSIIKS$^+$ plasmid. The plasmid, BK_E CAT, contained the regulatory region of human BK

virus in an early orientation (Pater and Pater, 1988). The human bcl-2 cDNA in pBSIIKS$^+$ was kindly provided as a gift from Dr. S.J. Korsmeyer. The adenovirus major late promoter (AdMLP) in pUC13 was kindly provided by Dr. Kotlo Kumar. The pO.7CAT, containing the murine p53 promoter upstream of CAT gene in pUC18 CAT, was a generous gift from Dr. D. Reisman (Reisman and Rotter, 1993).

2.2.6 Nuclear extract preparation

The method was described previously (Hennighausen and Lubon, 1987). Briefly, the confluent monolayer cells were washed three times with PBS. Cells were harvested by scraping with rubber policemen into 50 mL tubes. Cells were pelleted by centrifuging at 1,800 rpm for 10 min at 4^0 C. The cell pellet was washed two times with 10 volumes of PBS. The cell pellet was suspended in 5 volumes buffer A with 0.3 M sucrose (10 mM HEPES-NaOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF and 2 μg/mL each of protease inhibitors, like antipain, pepstatin, leupeptin and aprotinin). Cells were homogenized in a Dounce glass homogenizer with a B type pestle by applying 10-12 strokes. Later, NP-40 was added to a final concentration of 0.3-0.4% and homogenization was continued for another 1-2 strokes. Cell nuclei were pelletized by centrifugation at 2,700 rpm for 15 min at 4^0 C. Nuclei were washed two times with buffer A containing 0.3 M sucrose.

Next, the nuclei were resuspended in 2.5 volumes of

buffer B (400 mM NaCl, 10 mM HEPES-NaOH pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF and 5% glycerol). The nuclei were homogenized in Dounce glass homogenizer by applying exactly 10 strokes. The homogenized nuclei were stirred very gently at 4^0 C for 30 min. Then, lysates were dialysed against 50 volumes of buffer C (?0 mM HEPES-NaOH pH 7.9, 75 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, 20% glycerol and 0.5 mM PMSF) for 4 hrs at 4^0 C. Then, the dialysed lysates were centrifuged at 20,000 rpm for 1 hr at 4^0 C. The resultant supernatant was aliquoted, quick frozen and stored at -70^0 C. The protein concentration was determined using a kit from Bio-Rad according to the manufacturer's instructions.

2.2.7 Whole cell extract preparation

The protocol was adapted from Tasset et al (1990) with minor modifications. The confluent monolayers of cells were washed with PBS and scraped with rubber policemen into 50 mL centrifuge tubes. Cells were pelleted by centrifuging at 1,800 rpm for 10 min at 4^0 C. The cell pellet was washed three times with 10 volumes of chilled PBS. Then the cell pellet was suspended in 2.5 volumes of whole cell extract (WCE) buffer containing 10 mM Tris-HCl pH 7.6, 1mM EDTA pH 8.0, 400 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol, 1 mM PMSF and 2 µg/mL of each of protease inhibitors, like antipain, pepstatin, leupeptin and aprotinin. The cells were disrupted by repeated cycles of freezing in liquid nitrogen and thawing on ice. Cell debris

was pelleted by centrifugation for 1 hr at 39,000 rpm and 4^0 C. The resultant supernatant was aliquoted, quick frozen and stored at -70^0 C. The protein concentration was determined using a kit from Bio-Rad.

2.2.8 *In vitro* translation of wild-type and mutant *bag-2* cDNAs

The wild-type and mutant *bag-2* cDNAs in pBSIIKS$^+$ were used for coupled *in vitro* transcription and translation using a TNT *in vitro* translation kit from Promega. The cDNA was linearized by cleaving with NotI at its 3' end. The mutant cDNAs were made by digesting the wild-type *bag-2* cDNA with the restriction enzymes indicated in Fig. 2.1. One μg cDNA regulated from the T3 promoter was incubated in a reaction mixture containing 25 μL rabbit reticulocyte lysate, 2 μL TNT buffer, 1 μL T3 polymerase, 1 mM amino acid mixture, 10 mCi/mL [^{35}S]methionine, 0.5 mCi/mL [^{3}H]leucine, 40 U RNasin and 15 μL water. The incubation was carried out at 30^0 C for 1.5 hrs. Then, the resulting protein was stored at -20^0 C for subsequent use. The wild-type and mutant *bag-2* cDNAs were also nonradioactively translated with the complete amino acid mixture for DNA-binding assays.

2.2.9 Production of BAG-2 in bacteria

The *bag-2* cDNA from pBSIIKS$^+$ was liberated with SalI and NotI and cloned into the SalI and NotI sites of the pGEX-4T-1

vector. To ensure that the insert was cloned into the correct orientation, the 5' cloning site was sequenced using the 5'-p-GEX sequencing primer. The recombinant plasmid was transformed into E.coli BL21 cells.

Subsequent protocols for the induction and purification of fusion proteins were followed, as recommended by the manufacturer (Pharmacia). Finally, the concentration of recombinant BAG-2 was estimated using a kit from Bio-Rad.

2.2.10 Gel mobility shift assay

The sequences of oligonucleotides used were described previously (Kumar, 1994). The probes were prepared by nick translating the oligonucleotides in the presence of [α^{32}P]dCTP. The method for gel retardation was described by Chodosh *et al* (1988) with modifications. The *in vitro*-translated protein was used in the gel mobility shift assays. The binding reactions were done in 35 µL in a buffer containing 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 5 mM DTT, 150 mM KCl, 12% glycerol, 10 µg poly(dI.dC).poly (dI.dC), 15,000 cpm nick translated probe and 4 µL *in vitro*-translated protein. The reactions were incubated at RT for 30 min. The reaction products were resolved on 4% non-denaturing polyacrylamide gels at 100 V in 22 mM Tris-Borate, 0.5 mM EDTA, with buffer recirculation at 4^0 C. Gels were then dried and subjected to autoradiography.

2.2.11 Southwestern blot analysis

The method was described previously (Vinson et al., 1988) and is used here with several modifications. Briefly, NEs from UD, P19 glial and P19 muscle cells, HeLa cells and U87 MG cells were resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membranes in a semi-dry transfer apparatus (Bio-Rad). The filters were then blocked in binding buffer containing 5% non-fat Carnation dry milk powder in 25 mM HEPES-NaOH pH 7.9, 5 mM $MgCl_2$, 0.5 mM DTT and 25 mM NaCl overnight at 4^0 C. Following blocking in the milk powder, the filters were probed with 10^6 cpm/mL of nick-translated JCV NF-1 II/III oligonucleotide in binding buffer with gentle agitation at RT for 2 hrs. The membranes were washed three times with binding buffer 15 min each at RT. Membranes were air dried and subjected to autoradiography.

2.2.12 Far-Western blot analysis

This method is widely used to detect protein-protein interactions. The method was as described (Inostroza et al., 1992) with major modifications. Briefly, 50 µL in vitro-translated Bcl-2 protein was resolved on 12% SDS-PAGE gels and transferred to nitrocellulose membranes by semi-dry transfer. Later, the membrane was blocked in buffer A containing 20 mM HEPES-NaOH pH 7.9, 50 mM NaCl, 1 mM EDTA and 10 mM β-mercaptoethanol, with 5% non-fat Carnation dry milk powder at RT for 2 hrs with gentle agitation. Membranes were then incubated with radiolabelled in vitro-translated BAG-2 protein

in buffer B containing 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% NP-40, 2 mM DTT, 0.05% BSA and 5% glycerol for 4 hrs at RT. Membranes were then washed three times 15 min each with TENNS buffer (2.5 mM Tris-Hcl pH7.4, 2.5 mM EDTA, 250 mM NaCl, 1% NP-40 and 2.5% sucrose). Membranes were then dried and subjected to autoradiography.

2.2.13 RNA preparation and analysis

Total RNA was isolated using a kit from Promega, following the manufacturer's instructions. The RNA in aqueous solution was stored at -70^0 C for further use.

2.2.13.1 Northern blot analysis

The method followed was according to Sambrook et al (1989). A total of 25 μg RNA in 4.5 μL water was mixed with 1 μL 10X formaldehyde gel running buffer (0.2 M MOPS pH 7.0, 80 mM sodium acetate and 10 mM EDTA, pH 8.0), 3.5 μL formaldehyde and 10 μL of formamide. Following incubation at 65^0 C for 15 min, the RNA was resolved by electrophoresis on 1% agarose gels containing 2.2 M formaldehyde. Later, the RNA was transferred to nitrocellulose membranes by the capillary transfer method using 10X SSC (1X SSC is 0.15 M NaCl and 0.015 M Sodium Citrate). The membrane was then prehybridized in hybridization buffer containing 1% BSA, 1 mM EDTA, 0.43 M $NaH_2Po_4.H_2O$ and 7% SDS for 4 hrs at 65^0 C. The probes were made by nick translating the full length cDNA or the 3'-end of the cDNA, and γ-actin internal control in the presence of

[α^{32}P]dCTP. Probes were then denatured and added at 10^5 cpm/mL of hybridization buffer. The hybridization was continued overnight at 65^0 C.

Membranes were washed first with 2X SSC and 0.1% SDS at RT for 15 min, followed by two washes with 0.1X SSC, 0.1% SDS at 65^0 C for 5 min each. Membranes were then subjected to autoradiography.

2.2.13.2 Primer extension analysis

Primer extension analysis was done using a kit from Promega, according to the manufacturer's instructions. A total of 10 μg of total RNA was used. E.coli tRNA was used as a negative control. The extended products were resolved on a 6% polyacrylamide-8 M urea denaturing gel in Tris-borate-EDTA buffer. The dried gels were subjected to autoradiography.

2.2.14 *In vitro* transcription assay

The *in vitro* transcription technique was described by Kumar *et al* (1993), with certain modifications. Briefly, the plasmid JCV_ECAT was digested with PvuII to yield a 160 nt run-off transcript. One μg DNA template was incubated with either 75 μg WCE or WCE supplemented with 1 μg bacterially produced BAG-2 protein in a 50 μL volume containing 12 mM HEPES-NaOH pH 7.9, 12% glycerol, 60 mM KCl, 2 mM $MgCl_2$ and 1 mM each ATP, CTP, GTP and 0.5 mM [^{32}P]UTP. The reaction mixture was incubated at 30^0 C for 1-1.5 hrs. The reaction was stopped by adding 1.1 volumes stop buffer containing 80 mM EDTA, 1% SDS,

100 mM NaCl, 20 μg tRNA and 10 μg proteinase K. The samples were incubated in stop buffer for 30 min at 30^0 C. The samples were then extracted with phenol-chloroform-isoamylalcohol and ethanol-precipitated. The run-off transcripts were analyzed on 5% denaturing polyacrylamide-8M urea gels with ØX174 markers. The EcoRI-digested AdMLP in pUC13 was used as an internal control to yield a 400 nucleotide run-off transcript. The bands were quantitated by the Image Quant program (Molecular Dynamics).

2.2.15 Immunofluorescence assays

Indirect immunofluorescence was assayed according to the method of Harlow and Lane (1988). Briefly, the cells were grown overnight on cover slips and fixed with chilled acetone:methanol mixture (1:1) for 15 min at RT. After fixation, the cells were rehydrated with PBS for 10 min at RT. The cells were treated with 10% goat serum in PBS at RT for 30 min to block non-specific reactions. The cells were then probed with BAG-1 monoclonal antibodies in PBS at 0.1-2 μg/mL at RT for 1 hr. The cells were washed three times with PBS. The cells were then stained with anti-rabbit IgG FITC conjugate for 45 min at RT. After a final wash with PBS, cell staining was evaluated with a Leitz Diaplan microscope.

ERRATA

A sequence error had appeared in this thesis at page# 72. An " **A** " at position " **710** " should appear. Any inconvenience to this end is highly regretted. The author is thankful to Dr. Schang for analyzing the cDNA by using the Wisconsin genetics computer group sequencing package.

CHAPTER 3

RESULTS

Previous studies from our and other laboratories demonstrated that the glial cell-specific expression of JCV can be attributed to the *cis*-acting elements controlled by tissue-specific transcription factors. Using *in vitro* binding assays, it was suggested that NF-1 motifs may play a role in the glial cell-specific expression of JCV (Khalili *et al.*, 1988; Amemiya *et al.*, 1989; Nakshatri *et al.*, 1990a). Subsequently, the functional role of these NF-1 motifs was tested by site-directed mutagenesis. The results showed that these motifs are important for glial cell-specific expression of JCV (Kumar *et al.*, 1993). A cDNA was cloned in our lab, using the JCV NF-1 II/III probe from a P19 glial cell cDNA library. Preliminary work indicated that this cDNA transactivated both JCV_E and JCV_L. However, this cDNA was not fully characterized. This study addressed the characterization of this cDNA.

3.1 Sequence analysis of *bag-2* cDNA

As a first step in characterizing the cDNA, the sequence of the cDNA was determined. To this end, the cDNA was inserted into pBSIIKS$^+$ for sequencing. Both strands of the cDNA were sequenced twice. The full-length cDNA sequence and the deduced

Fig. 3.1. Nucleotide sequence and deduced amino acid sequence of mouse bag-2 cDNA.

The protein sequence is designated beginning with position 1 for the translation start codon ATG and stopping at the position 229 TGA termination codon. The first ATG is in bold face italics. The stop codon is singly underlined. The putative polyadenylation signal is bold face italicized and doubly underlined. The splice site consensus flanking sequence for a putative alternative exon is in bold face and doubly underlined. The three possible initiation methionines are shown in bold. For the first ATG, the immediately upstream 7 nucleotides that match the 12 nucleotide consensus sequence are shown in bold. The numbers on the left indicate nucleotides and the numbers on the right indicate the position of amino acids. Amino acids are indicated by the single letter code.

```
1      CCCACGCGTCCGGTGACCCGTAGCAAGAACGTGACCGGGACCCAGGTAGAGGAGGTGACC
61     AAGATCGAGGAGGCGACCCAAACCGAGGAAGTAACTGTGGCAGAAGAGGTGACCCAGACC

          M  A  K  T  E  E  M  V  Q  T  E  E  M  E  T  P  R  L          18
121    GACAACATGGCCAAGACCGAGGAGATGGTCCAGACGGAGGAAATGGAAACACCCAGACTC

       S  V  I  V  T  H  S  N  E  R  Y  D  L  L  V  T  P  Q  Q  G       38
181    AGCGTGATCGTCACCCACAGCAATGAGAGGTATGACCTTCTTGTTACCCCACAGCAAGGT

       N  S  E  P  V  V  Q  D  L  A  Q  L  V  E  E  A  T  G  V  P       58
241    AACAGTGAGCCAGTTGTCCAAGACTTGGCTCAGCTTGTTGAAGAGGCCACAGGAGTTCCA

       L  P  F  Q  K  L  I  F  K  G  K  S  L  K  E  M  E  T  P  L       78
301    CTACCTTTTCAGAAGCTCATATTTAAGGGAAAATCTCTGAAAGAAATGGAAACACCGTTG

       S  A  L  G  M  Q  N  G  C  R  V  M  L  I  G  E  K  S  N  P       98
361    TCAGCACTTGGAATGCAAAATGGTTGCCGAGTCATGTTAATTGGTGAAAAGAGCAATCCA

       E  E  E  V  E  L  K  K  L  K  D  L  E  V  S  A  E  K  I  A      118
421    GAAGAAGAGGTTGAGTTAAAGAAGCTGAAAGATTTGGAGGTATCTGCAGAGAAGATAGCT

       N  H  L  Q  E  L  N  K  E  L  S  G  I  Q  Q  G  F  L  A  K      138
481    AACCACCTGCAAGAATTGAATAAAGAGCTTTCTGGCATCCAGCAGGGTTTTCTGGCTAAG

       E  L  Q  A  E  A  L  C  K  L  D  R  K  V  K  A  T  I  E  Q      158
541    GAATTGCAAGCGGAGGCTCTCTGCAAACTTGATAGGAAAGTAAAAGCAACAATTGAGCAA

       F  M  K  I  L  E  E  I  D  T  M  V  L  P  E  Q  F  K  D  S      178
601    TTCATGAAGATCTTGGAGGAGATTGACACAATGGTCCTACCAGAACAATTTAAAGACAGC

       R  L  K  R  K  N  L  V  K  K  V  Q  V  F  L  A  G  V  T  Q      198
661    AGGCTAAAAAGGAAGAATTTGGTGAAAAAGGTTCAGGTGTTCTTAGCAGGTGTGACACAG

       W  S  N  T  S  A  K  R  Q  S  G  C  S  L  Q  T  W  P  W  L      218
721    TGGAGCAATACATCTGCCAAGAGACAGAGCGGCTGCAGTCTACAAACTTGGCCCTGGCTG

       N  E  V  Q  W  R  V  A  V  L  A  End                            229
781    AATGAAGTGCAGTGGAGAGTGGCTGTACTGGCCTGAAGAGCAGCTTTACAGCCCTGCCCT
841    CTCTGGAACAGAAGTCGCCTGTTTCTCCATGGCTGCCAGGGGCAACTAGCCAAATGTCAA
901    TTTCCCTGCTCCTCCGTCGGTTCTCAATGAAAAAGTCCTGTCTTTGCAACCTGAAAAAAA
961    AAAAAAAAAAAAA
```

protein sequence are shown in Fig. 3.1. The cDNA has intact 5' and 3' ends. Since the 5' end contains the ATG start codon and 3' end contains the TGA stop codon, the cDNA sequence represents an mRNA with a 126 nucleotide 5'- possible untranslated region and a 156 nucleotide 3'-untranslated region. The first ATG at nt 127 is followed by a long ORF of 687 nucleotides, which extends to nt 813. The first ATG is followed by two more ATGs at nts 145 and 163. The methionine at nt 127 is likely to be the start site, since 7 of 12 nucleotides immediately adjacent to the ATG match the consensus 5'-noncoding sequence, (GCC)GCCA/GCCATGG, for initiation of translation in vertebrates (Kozak, 1987).

The stop codon is at nt 814. A putative polyadenylation signal, AATGAAA is at nt 925. The poly(A) signal is usually seen 15-25 nucleotides upstream of the poly(A) tail in eukaryotes and is important for cleavage and for the formation of the poly(A) tail (Proudfoot, 1991).

3.2 Structural features of the deduced amino acid sequence

Complete sequencing of the cDNA revealed one large ORF coding for a protein of 229 amino acids, with an apparent molecular weight of 30.6 kDa.

The computerized analysis of cDNA through the NCBI blast server with the sequences reported in GenBank (release 89) showed a high degree of homology to a protein called BAG-1, for Bcl-2 associated athanogene-1 (Takayama et al., 1995)

Fig. 3.2. Protein sequence homology between BAG-1 and BAG-2.

The deduced amino acid sequence is aligned with BAG-1 amino acid sequence to show maximum homology. Identical amino acid sequences are linked with vertical bars. The numbers indicate the position of amino acids.

```
BAG-1  1    MAKTEEMVQTEEMETPRLSVIVTHSNERYD  30
            ||||||||||||||||||||||||||||||
BAG-2  1    MAKTEEMVQTEEMETPRLSVIVTHSNERYD  30

BAG-1  31   LLVTPQQGNSEPVVQDLAQLVEEATGVPLP  60
            ||||||||||||||||||||||||||||||
BAG-2  31   LLVTPQQGNSEPVVQDLAQLVEEATGVPLP  60

BAG-1  61   FQKLIFKGKSLKEMETPLSALGMQNGCRVM  90
            ||||||||||||||||||||||||||||||
BAG-2  61   FQKLIFKGKSLKEMETPLSALGMQNGCRVM  90

BAG-1  91   LIGEKSMPEEEVELKKLKDLEVSAEKIANH  120
            ||||||||||||||||||||||||||||||
BAG-2  91   LIGEKSMPEEEVELKKLKDLEVSAEKIANH  120

BAG-1  121  LQELMKELSGIQQGFLAKELQAEALCKLDR  150
            ||||||||||||||||||||||||||||||
BAG-2  121  LQELMKELSGIQQGFLAKELQAEALCKLDR  150

BAG-1  151  KVKATIEQFMKILEEIDTNVLPEQFKDSRL  180
            ||||||||||||||||||||||||||||||
BAG-2  151  KVKATIEQFMKILEEIDTNVLPEQFKDSRL  180

BAG-1  181  KRKNLVKKVQVFLAECDTVEQYICQETERL  210
            ||||||||||||||
BAG-2  181  KRKNLVKKVQVFLAGVTQWSNTSAKRQSGC  210

BAG-1  211  QSTNLALAE                       219

BAG-2  221  SLQTWPWLNEVQWRVAVLA             229
```

(Fig. 3.2). Hence, I named the cDNA bag-2. The accession number for the BAG-1 is U17162. The bag-1 cDNA was isolated from a mouse embryonic cDNA library, with the intention of finding protein(s) that could interact with and mediate the anti-apoptotic effects of Bcl-2 by the yeast two-hybrid system. Bcl-2 is a proto-oncogene, and is essential for protecting the cells from apoptosis, as discussed in the Introduction. The cloned BAG-1 was shown to interact with Bcl-2. Furthermore, in gene transfer studies, it protected cells from apoptosis (Takayama et al., 1995).

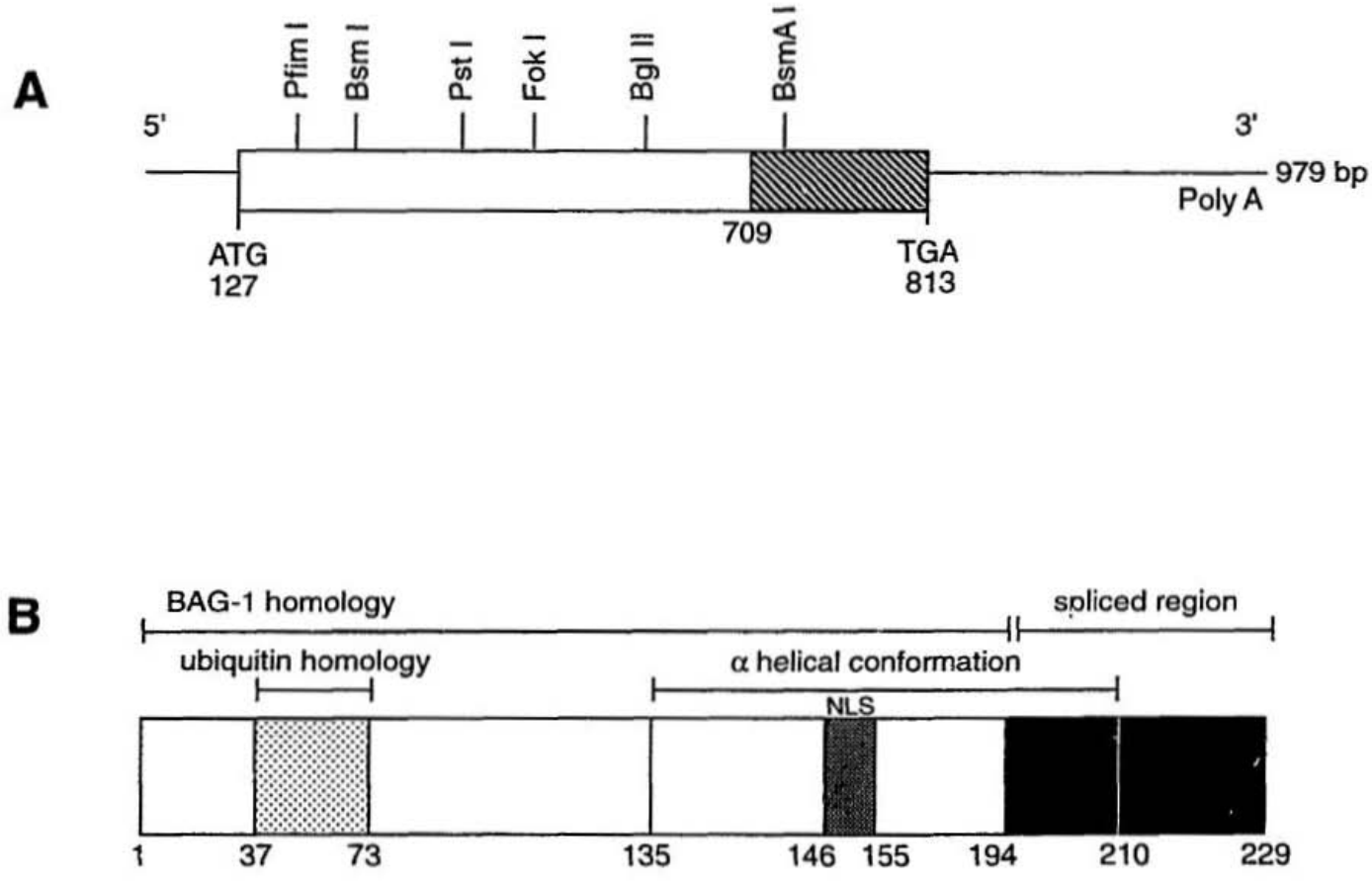
The homology at the nucleotide level starting from 1st ATG at nt 127 to nt 709, bag-2 is identical to bag-1 (data not shown). Similarly, at the protein level from a.a 1 to a.a 194, BAG-2 is identical to BAG-1; from 195 a.a to the end a different amino acid sequence was observed in BAG-2 (Fig. 3.2). The nt at the point these two sequences diverge is a splice site with AG at the 5' and GT at the 3' end (Fig. 3.1; Jackson, 1991). Thus, BAG-2 appears to be different from BAG-1 at its C-terminus (from a.a 195 to a.a 229). The BAG-2 is an acidic protein with a pI of 4.81, while the pI of BAG-1 is 4.94. One interesting feature of BAG-2 is the presence of multiple glutamic acid (E) residues (35 of 229).

A limited amount of homology was observed with the several ubiquitin-like proteins and other functionally uncharacterized human cDNA clones. Further, structural analysis of BAG-2 for the hydropathy index (Kyte and

Fig. 3.3. Structural details of bag-2 cDNA and BAG-2 protein (the figure is not drawn to scale).

A. Structural details and partial restriction map of the bag-2 cDNA . The boxed area represents the open reading frame. Solid lines represent the flanking 5' and 3' untranslated regions. A partial list of enzymes that cleave bag-2 ORF one time are indicated. Shaded area represents the putative alternatively spliced 3'-end. The figure is not drawn to scale.

B. Structural features of mouse BAG-2 protein. Numbers at the bottom represent positions of amino acids in BAG-2. The α-helical, BAG-1 and ubiquitin (dotted box) homology regions are indicated. The C-terminus region that differs from that of BAG-1 was shown as a blackened box. The putative nuclear localization signal (NLS) is shown between amino acids 146-155, as indicated by a stippled box.


A
Pfim I
Bsm I
Pst I
Fok I
Bgl II
BsmA I
5'
3'
979 bp
Poly A
ATG
127
709
TGA
813
B
BAG-1 homology
spliced region
ubiquitin homology
α helical conformation
NLS
1
37
73
135
146
155
194
210
229

Doolittle, 1982) suggested that this protein is amphipathic. The region between a.a 135 to 210 of the BAG-2 appears to have an α-helical conformation, according to the COIL program of P/C gene (Fig. 3.3). The nuclear localization signal (a.a 146-155) that is partially identical to that of c-myc (Dang and Lee, 1988) is indicated by a stippled box in Fig. 3.3. B.

3.3 BAG-2 protein subcellular distribution

Since Bcl-2 is a mitochondrial protein, and BAG-1 interacts with Bcl-2 to mediate its anti-apoptotic effects (Takayama et al., 1995), it is possible that BAG-1 is a cytoplasmic protein. Since BAG-2 is 85% identical to BAG-1 , it is also possible that BAG-2 is a cytoplasmic protein. However, previous preliminary study suggested that BAG-2 is a transcription factor (Kumar, 1994). To act as a transcription factor, BAG-2 should therefore be a nuclear protein. Detailed analysis of the BAG-2 amino acid sequence revealed a putative nuclear localization signal (Fig. 3.4, 1) that is partially identical to the human c-myc NLS (Dang and Lee, 1988). The NLS between amino acids 146-155 was shown to have an arginine and two lysines, that are important for translocation in c-myc (Dang and Lee, 1988).

3.3.1 Immunofluorescence analysis

To identify the intracellular location of BAG-2, immunofluorescence analysis was done in P19 glial cells, which

Fig. 3.4. Sequence and function of BAG-2 nuclear localization signal.

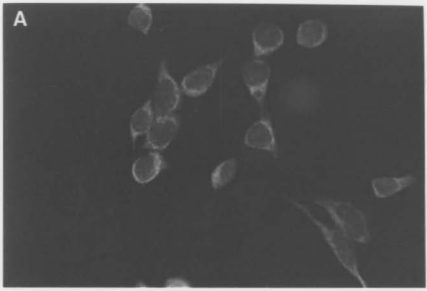
1. Location of NLS sequence. The NLS that is partially identical to the NLS observed in human c-myc, between amino acids 146-155 is shown in bold face italics. The doubly underlined arginine and lysines were shown to be critical in nuclear translocation in human c-myc. The numbers indicate the position of amino acids.

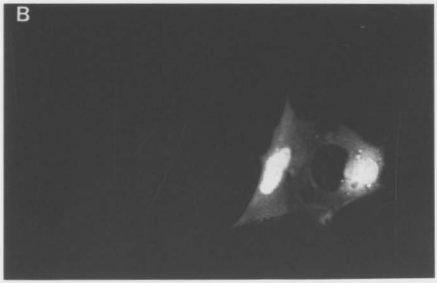
2. Immunofluorescene analysis of BAG-2 subcellular location. Panels show immunofluorescene in UD (panel A) and P19 glial (panel B) cells. Please note the cytoplasmic UD, and the cytoplasmic and intranuclear P19 glial immunofluorescence. The fluorescence is also enhanced in glial cells relative to UD cells.

1

```
MAKTEEMVQTEEMETPRLSVIVTHSNERYD     30
LLVTPQQGNSEPVVQDLAQLVEEATGVPLP     60
FQKLIFKGKSLKEMETPLSALGMQNGCRVM     90
LIGEKSNPEEEVELKKLKDLEVSAEKIANH    120
LQELNKELSGIQQGFLAKELQAEALCKLDR    150
KVKATIEQFMKILEEIDTMVLPEQFKDSRL    180
KRKNLVKKVQVFLAGVTQWSNTSAKRQSGC    210
SLQTWPWLNEVQWRVAVL                229
```

2





were functional for JCV expression. UD cells were used as a negative control since these cells were not functional for JCV expression (Nakshatri et al., 1990a). The analysis used a BAG-1 polyclonal antibody which would be expected to cross-react with BAG-2. In UD cells, the BAG-2 protein was found exclusively in the cytoplasm (Fig. 3.4, 2A). In P19 glial cells, BAG-2 protein was found in the cytoplasm where protein synthesis is known to occur, but was found mainly in the nucleus (Fig. 3.4, 2B). All P19 glial cells did not show the nuclear immunofluorescence. Since differentiation with retinoic acid yields a mixture of brain cells, at present it is not clear in which type of cells BAG-2 protein is nuclear. However, of the cells with nuclear immunofluorescence, 75% are glial cells when stained with antibody to the GFAP marker protein. The amount of fluorescence was intense in P19 glial cells than in UD cells, which is consistent with the expression of BAG-2 in these cells only (see Section 3.5.4). The immunofluorescence experiments were repeated three times and the same results were consistently observed. Taken together, these results indicate that BAG-2 protein was translocated into the nucleus only in P19 glial cells. Interestingly, BAG-2 was observed in the cytoplasm of muscle cells and in the cytoplasm as well as in the nuclei of HeLa cells (data not shown). NOTE: Since this immunofluorescence analysis used a BAG-1 antibody, it is possible that both BAG-1 and BAG-2 or BAG-2 alone might have been detected in UD and

P19 glial cells. Therefore, the subsequent experiments were designed to delineate the subtle differences between BAG-1 and BAG-2.

3.4 Tissue-specific expression of BAG-2

3.4.1 Northern blot analysis of total RNA from P19, HeLa and U87 MG cells

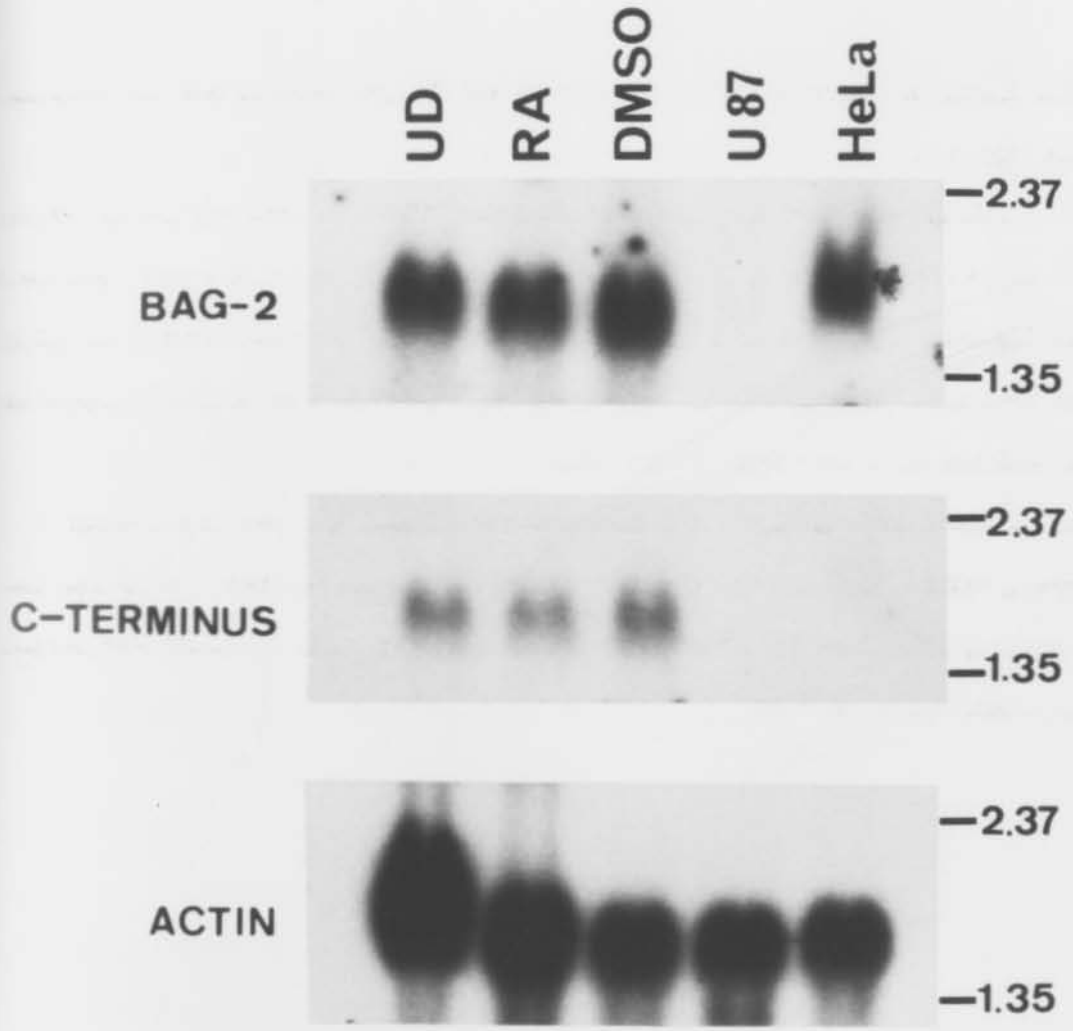
JCV expresses and replicates efficiently in brain cells (Kenney et al., 1984) and the NF-1 sites in the JCV regulatory region were shown to be critical for glial cell-specific expression (Kumar et al., 1993). The bag-2 cDNA, cloned using a NF-1 II/III probe from a P19 glial cell cDNA library, was shown to be expressed at higher levels in P19 glial cells, than in UD cells (Kumar, 1994). To know whether bag-1 and possibly bag-2 are ubiquitously expressed or restricted to certain cell types, total RNA from mouse cells, for instance P19 UD, glial and muscle cells, and human cells, like HeLa cells and U87 MG cells was probed on Northern blots with either full length bag-2 cDNA or its 3'-end as probes (Fig. 3.5).

The 1.4 kb and 1.6 kb bag-2 mRNAs were detected in UD, P19 glial, P19 muscle and HeLa cells, respectively. Surprisingly, no bag-2 mRNA was detected in U87 MG cells (Fig. 3.5, upper panel). Though U87 MG cells are derived from a human glioblastoma and support JCV expression, it is surprising to note that BAG-2 is not expressed in these cells. A possible

Fig. 3.5. Northern blot analysis of bag-2 RNA from different cell types.

Total RNA (20 μg) isolated from the indicated cell lines was separated on a 1% agarose and 0.66 M formaldehyde gel and hybridized with ^{32}P-labelled bag-2, bag-2 3'-end (nt 735-973) and γ-actin probes. Molecular weight markers are indicated on the right and are given in kb.

The probes are indicated and their sizes are as follows: bag-2, 973 bp; bag-2 3'-end (indicated as C-terminus in the figure), 214 bp 3' fragment from bag-2; Actin, 2.2 kb BamHI fragment of γ-actin.


UD
RA
DMSO
U87
HeLa
BAG-2
C-TERMINUS
ACTIN
2.37
1.35
2.37
1.35
2.37
1.35

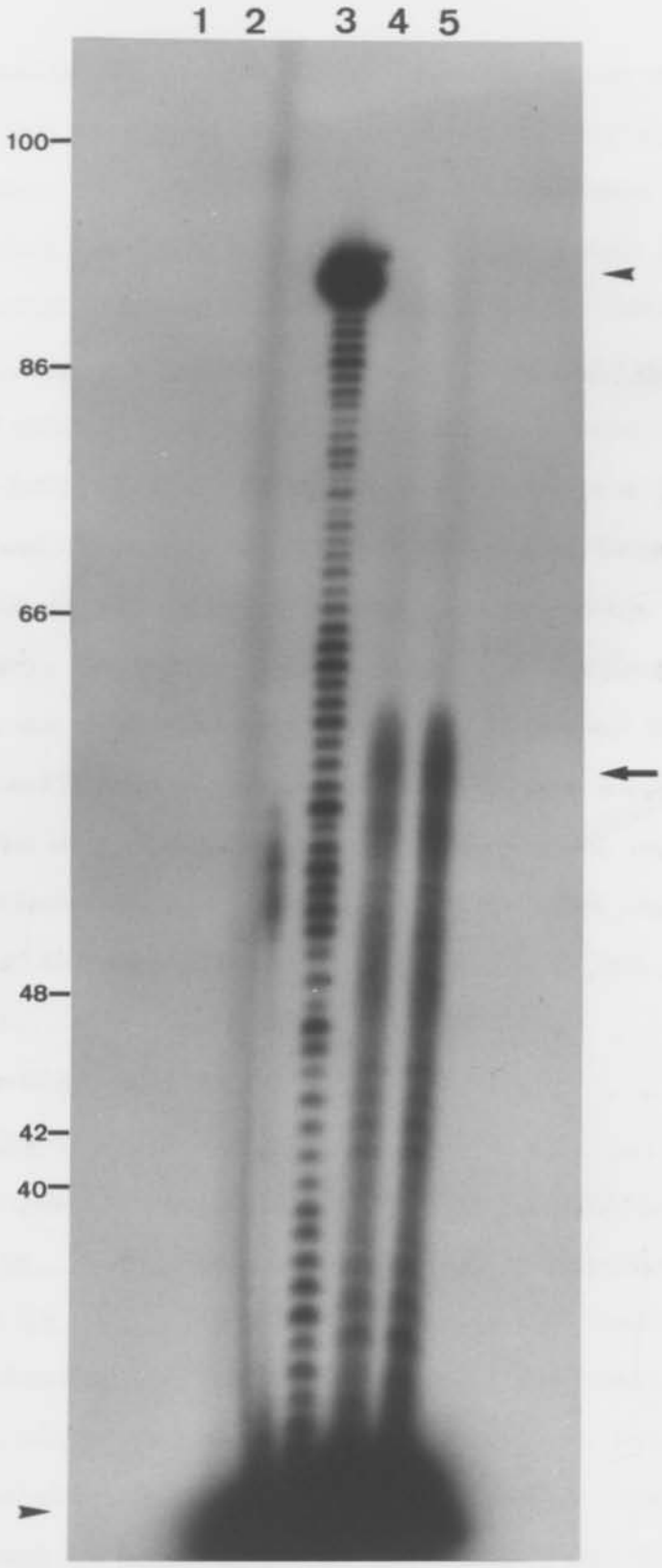
explanation for this is since these cells contain several chromosomal deletions (Ponteen and MacIntyre, 1968), it is possible that the gene which codes for BAG-2 has been lost. This possibility could be tested in Southern blot analysis with bag-2 as a probe. Interestingly, the size of the bag-2 mRNA in HeLa cells was approximately 200 nt longer than bag-2 mRNA from UD, P19 glial and P19 muscle cells (Fig. 3.5, upper panel). The bag-2 mRNA size differences were consistently observed between HeLa and P19 cells in 4 independent experiments. Furthermore, results with the 3'-end (nt 735-973) of bag-2 as a probe revealed the expression of this region only in P19 cells, but not in HeLa cells (Fig. 3.5, middle panel). Therefore, it appears that this portion is spliced only in P19 cells. From these results it is clear that BAG-1 is present in UD, P19 glial, P19 muscle and HeLa cells, whereas the BAG-2 C-terminus (a.a 195-229) is restricted to only P19 cells. The unique and longer mouse protein was reminiscent of another transcription factor which plays a central role in the regulation of gene expression at the transcriptional level. This transcription factor is TBP (Zawel and Reinberg, 1993).

3.4.2 5'-end analysis of RNA from unique cell types

The Northern blot analysis revealed a 1.4 kb mRNA for BAG-2, but the size of the cDNA was only 973 bp. Therefore, one possibility is that the presence of extra 5' sequences.

Fig. 3.6. Primer extension analysis of RNA from P19 glial and UD cells.

Total RNA (50 μg) from UD (lane 4), P19 glial (lane 5) cells was hybridized to an oligonucleotide probe derived from the bag-2 cDNA at nt 57-85. The hybrids were treated with avian myeloblastosis reverse transcriptase under conditions recommended by the supplier and the products were resolved by electrophoresis through a 6% polyacrylamide/7 M urea gel. The arrow illustrates the position of the major 56 nucleotide bag-2 extension product. Lanes 1 and 2 represent primer extension analysis using no RNA and E.coli tRNA, respectively. Lane 3 is a kanamycin RNA positive control, supplied by the manufacturer. The arrowhead indicates the 88 nucleotide extended product obtained using the primer supplied along with the kanamycin RNA. The molecular weight markers in bases are indicated on the left.


1 2 3 4 5
100
86
66
48
42
40

The other possibilities are either the presence of extra 3' sequences or the presence of other alternatively spliced bag-2-related mRNAs. To further map the 5' terminus precisely, primer extension analysis was done with total RNA from UD and P19 glial cells. A synthetic 24-mer probe was designed corresponding to sequences at nt 57-85 of bag-2 cDNA. Elongation of this primer resulted in a 56 nucleotide fragment with RNA from both UD and P19 glial cells as the major species and a number of lesser and smaller species. This indicated that the 5' end of the bag-2 mRNA was genuine (Fig. 3.6, lanes 4 and 5, arrow). Beyond 56 nucleotides, no further extension was observed. As a negative control, I employed E.coli tRNA (Fig. 3.6, lane 2) and a positive control was kanamycin mRNA supplied by the manufacturer (Fig. 3.6, lane 3, arrowhead on the right). Primer extension analysis with total RNA from HeLa cells showed a 400 nt extension product (data not shown).

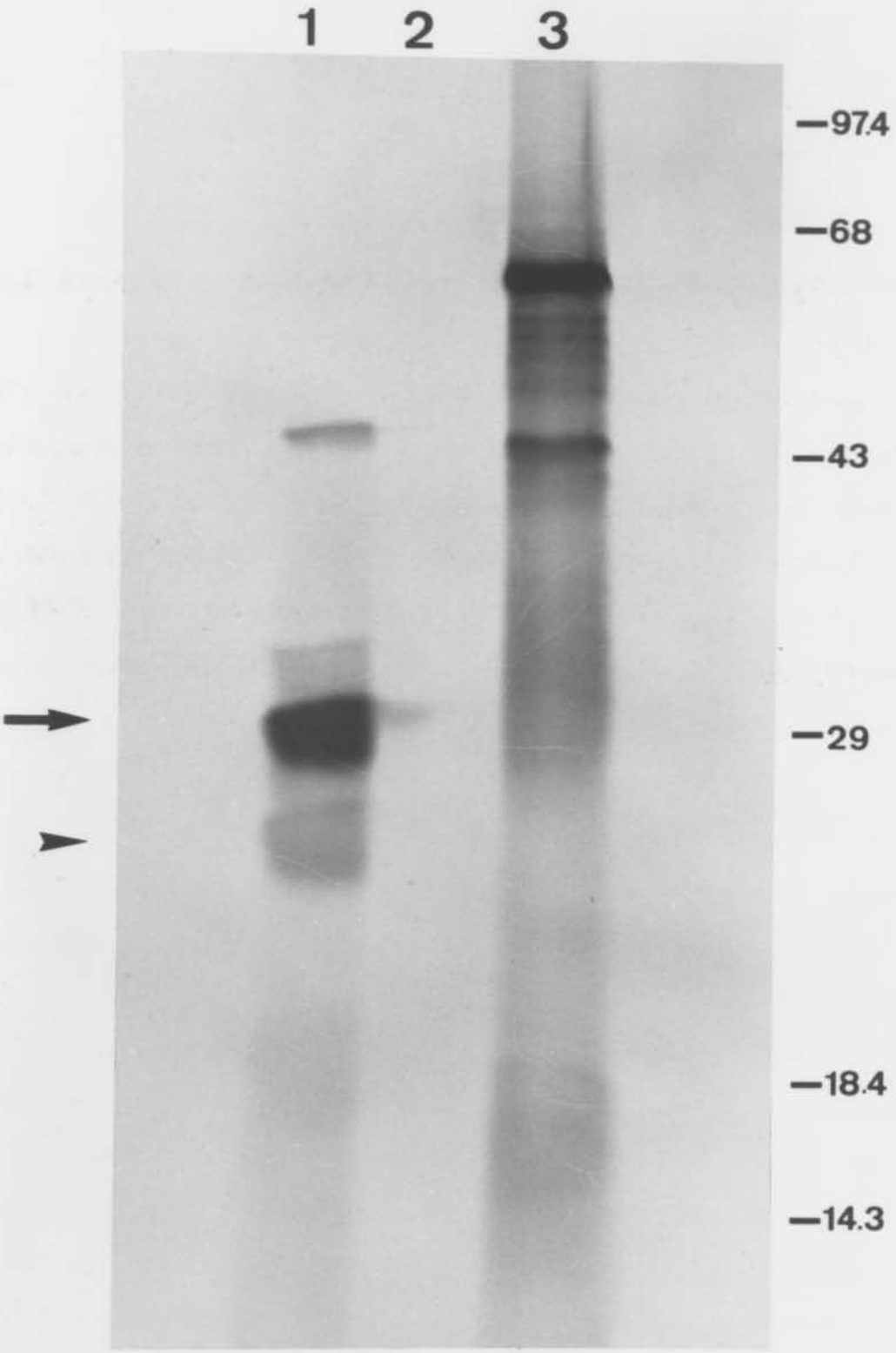
3.5. *In vitro*-binding studies of BAG-2

3.5.1 *In vitro*-translation of bag-2 cDNA

To study the DNA-binding properties of BAG-2, the bag-2 cDNA was *in vitro*-transcribed and translated. The radiolabelled *in vitro*-translated protein was resolved on an SDS-PAGE denaturing gel and subjected to autoradiography to determine the sizes and the number of species. Surprisingly, *in vitro* translation showed two bands, a major band of 30 kDa and a minor band of approximately 28.5 kDa (Fig. 3.7, lane 1,

Fig. 3.7. SDS-PAGE analysis of *in vitro*-synthesized BAG-2 protein.

The ^{35}S-labelled proteins were synthesized using a kit from Promega with conditions recommended. Lanes were: 1, *bag-2* cDNA (1 μg). The arrow indicates the major 30 kDa protein and the arrowhead shows the minor 28.5 kDa protein; 2, no DNA and 3, luciferase control DNA (1 μg). Luciferase is a 61 kDa protein. Markers on the right are indicated in kDa.


1
2
3
—97.4
—68
—43
—29
—18.4
—14.3

arrow and arrowhead, respectively). Since there are 3 ATGs at the 5' end of the cDNA, alternative use of these ATGs might be occurring. Since a major band was observed at 30 kDa for BAG-2, it is likely that the initiation of translation occurred from the first ATG. This interpretation is further supported by the recent finding that translation is restricted to the first ATG when the second ATG follows the first one closely (Kozak, 1995). Most likely, the 28.5 kDa protein arises from the 3rd ATG, based on the expected molecular weight. *In vitro* translation in the absence of cDNA did not reveal any protein (Fig. 3.7, lane 2). The positive luciferase control DNA, yielded the expected 61 kDa protein (Fig. 3.7, lane 3). The 42 kDa protein might have arisen from the background labelling of the reticulocyte lysate by ^{35}S-methionine in the *in vitro* translation reaction (Jackson and Hunt, 1983).

3.5.2 Mobility shift assays of BAG-2 protein probed with JCV NF-1 II/III oligonucleotide

To study the DNA-binding properties of the BAG-2, the unlabelled *in vitro*-translated protein was used in mobility shift assays. The wild-type and mutant NF-1 region II/III oligonucleotides used as probe and competitors, were described previously (Kumar *et al.*, 1993). Only the *in vitro*-translated protein was able to significantly retard the mobility of the JCV NF-1 II/III oligonucleotide. This binding could be competed with a 200-fold excess of unlabelled wild-type

Fig. 3.8. Specific *in vitro* binding of BAG-2 to NF-1 II/III oligonucleotide in gel mobility shift assays.

Assays were done using a nick-translated double-stranded NF-1 II/III oligonucleotide as a probe and 4 μL each of *in vitro*-translated BAG-2, reticulocyte lysate and *in vitro*-translated luciferase protein.

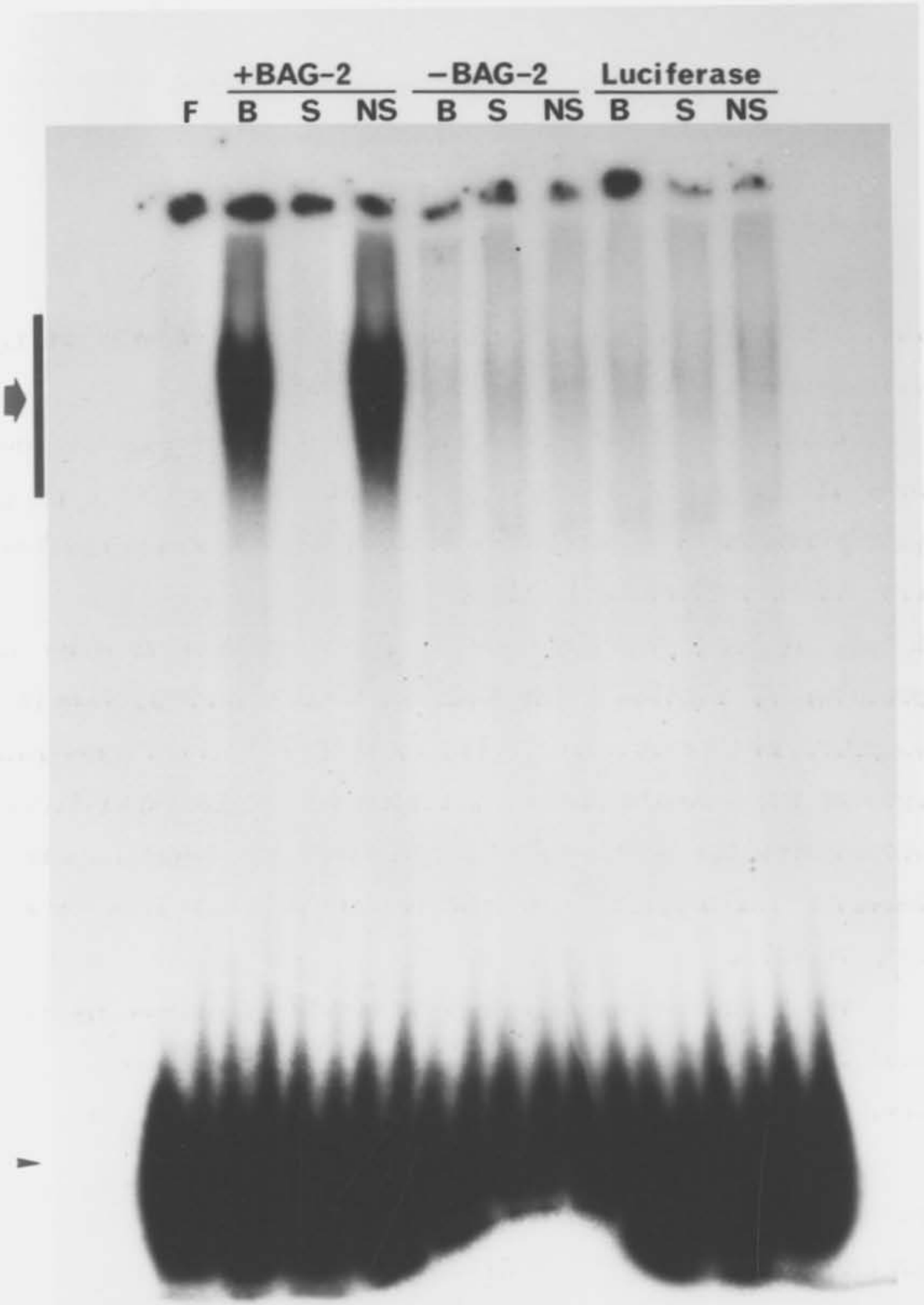
Lanes: F, free oligonucleotide probe in the absence of protein; B, binding assay with no competitor; S, specific competition by homologous (wild-type) competitor in 200-fold excess; NS, non-specific competition by mutated NF-1 II/III oligonucleotide in 200-fold excess. Broad arrow and arrowhead indicate low-mobility DNA-protein complex and free probe, respectively.

The sequences of wild type NF-1 II/III oligonucleotide used as a probe and the specific (S) competitor were:

NF-1 II/III
5'-GTCGACTGGCTGCCAGCCAA-3'
3'- ACCGACGGTCGGTTGTCGAC-5'.

The mutant NF-1 oligonucleotide as a non-specific (NS) competitor was:

mutant NF-1 II/III
5'-GGAGTACTGCCAGACCAG-3'
3'- CATGACGGTCTGGTCCCT-5'.


+BAG-2
−BAG-2
Luciferase
F
B
S
NS
B
S
NS
B
S
NS

oligonucleotide. However, the same amount of a mutated NF-1 II/III oligonucleotide had no effect, indicating that the complex observed was specific to the JCV NF-1 II/III oligonucleotide (Fig. 3.8, +BAG-2 lanes). No specific binding was observed with rabbit reticulocyte lysate alone (Fig. 3.8, -BAG-2 lanes). A non-specific protein, luciferase, also showed no binding to the JCV NF-1 II/III oligonucleotide (Fig. 3.8, luciferase lanes). All these results were reproducible for at least three times. The background binding observed might have arisen from the NF-1 or NF-1-like proteins present in the reticulocyte lysate (Mermod et al., 1989).

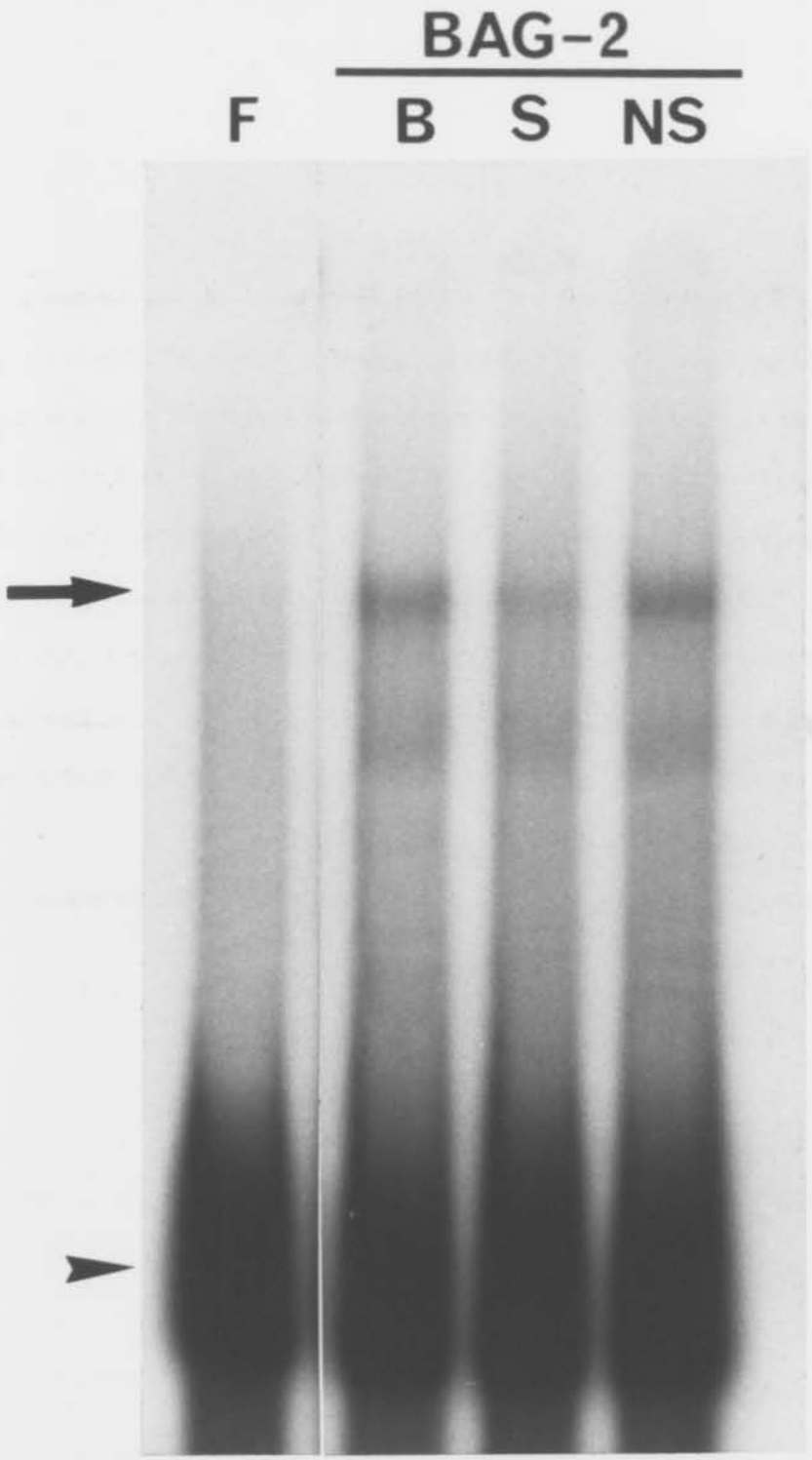
3.5.3 Mobility shift assays of BAG-2 protein probed with JCV enhancer

To test the binding of BAG-2 in the context of the intact JCV enhancer and to re-evaluate the binding observed in Fig. 3.8, an end-labelled JCV enhancer was used as a probe in mobility shift assays. Again, in vitro-translated BAG-2 showed a specific binding to the JCV enhancer, and a 200-fold excess of the wild-type NF-1 II/III oligonucleotide substantially decreased the binding. However, a similar excess of the mutant NF-1 II/III oligonucleotide had no effect (Fig. 3.9, BAG-2 lanes). The presence of another band may indicate the binding of NF-1 or NF-1-related proteins present in the reticulocyte lysate (Mermod et al., 1989) to the JCV enhancer. Taken together, these DNA-binding assay results suggest that BAG-2

Fig. 3.9. *In vitro* binding of BAG-2 to JCV enhancer.

Mobility shift assays used the pUC19-JC plasmid containing the JCV enhancer at XbaI site. The JCV enhancer was liberated by cleaving with SalI and NotI restriction enzymes and end-labelled at SalI site with [α^{32}P]dCTP. This was used with 4 μL of *in vitro*-translated BAG-2 protein. Lanes: F, is a free probe with no protein; B, binding assay with protein; S, Specific competition with 200-fold excess wild-type NF-1 II/III competitor; NS, Non-specific competition with 200-fold excess mutant NF-1 II/III oligonucleotide.

The arrow and arrowhead indicate the specific complex and free probe, respectively.


BAG-2
F
B
S
NS

interacts specifically with the NF-1 motifs in the NF-1 II/III oligonucleotide and in the JCV enhancer.

3.5.4 Southwestern blot analysis

3.5.4.1 Southwestern blot analysis of NEs from P19 glial, P19 muscle, HeLa and U87 MG cells

To characterize the relative amount of BAG-2 protein in the nucleus more quantitatively, Southwestern blots with NEs from P19 glial, P19 muscle, HeLa and U87 MG cells were analyzed. Proteins of 30 kDA and 28.5 kDa were detected in NEs from P19 glial, P19 muscle and HeLa cells, but not in U87 MG cells, suggestive of BAG-2 and its isoform (Fig. 3.10). However, these results are in agreement with my Northern blot analysis (Fig.3.5), in which the mRNA for BAG-2 was not detected in U87 MG cells. Only a weak band at 30 kDa was observed in P19 muscle cells. Interestingly, only a very faint band at 30 kDa was detected in UD cells (data not shown). Another band in Southwestern blot assays is an 88 kDa protein present in both glial and non-glial cells (Fig. 3.10). This was demonstrated previously and was believed to be a TATA-like factor (Khalili *et al.*, 1988). All these bands were reproducibly observed for 3 times.

3.5.4.2 Southwestern blot analysis of *in vitro*-translated BAG-2

Southwestern blot analysis of BAG-2 protein that was *in*

Fig. 3.10. Southwestern blot analysis of nuclear extracts probed with JCV NF-1 II/III oligonucleotide.

The nuclear extracts from P19 glial, P19 muscle, U87 MG and HeLa cells were resolved on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane and probed with nick-translated double-stranded NF-1 II/III oligonucleotide. The small arrowhead indicates a ubiquitous 88 kDa protein. The arrows indicate the 30 and 28.5 kDa forms of BAG-2 protein. Molecular weight markers are indicated on right in kDa.

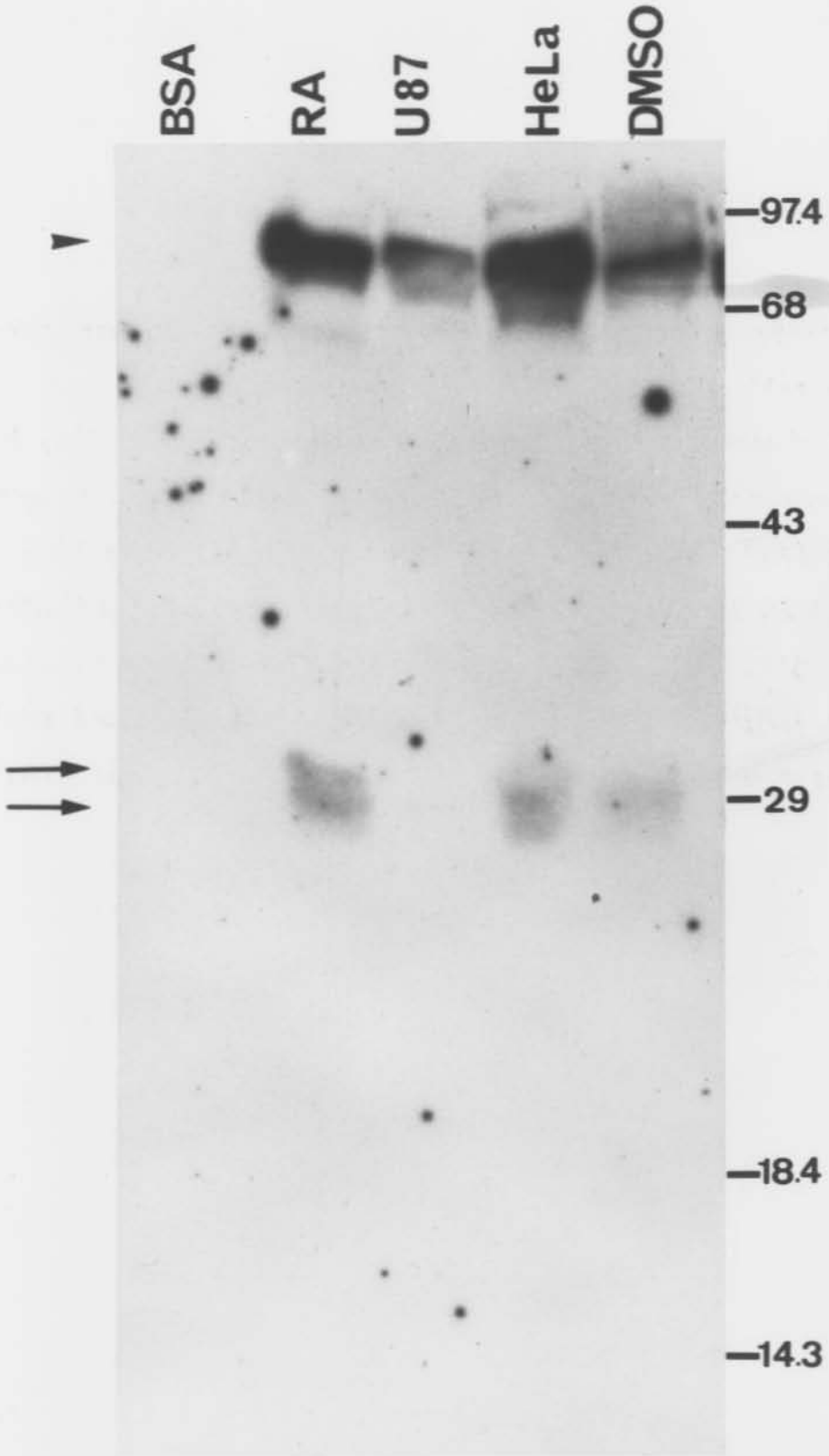
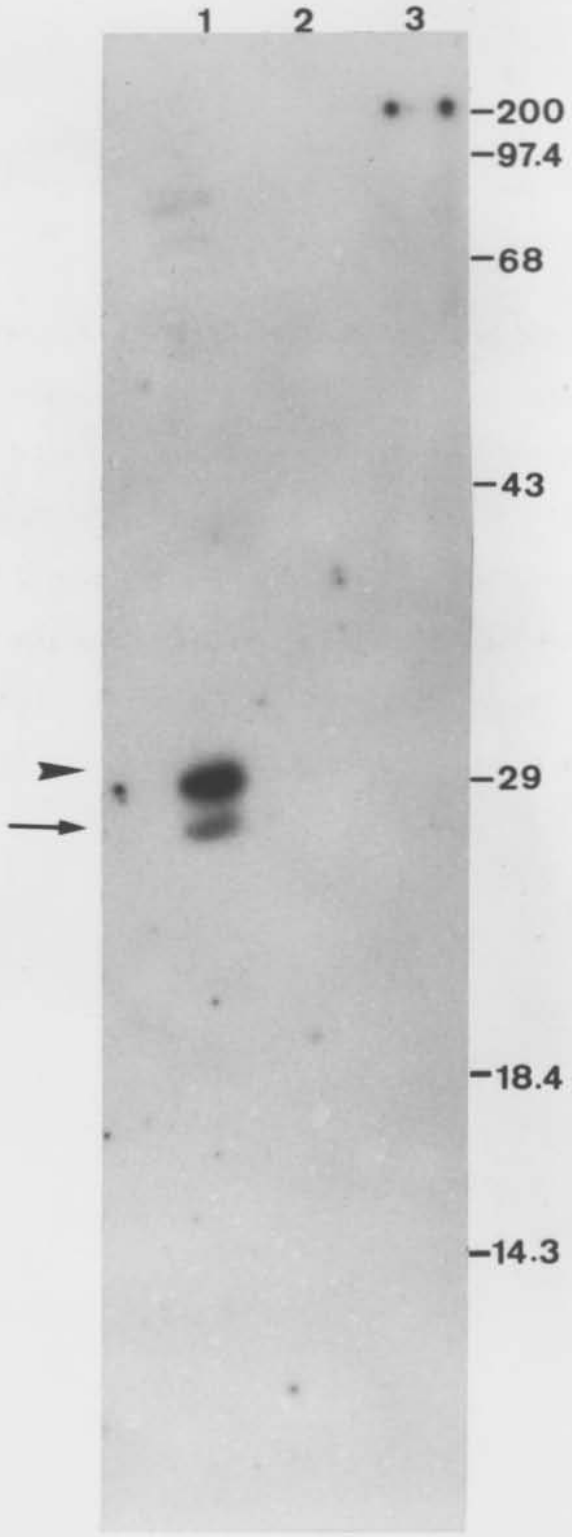

BSA
RA
U87
HeLa
DMSO
97.4
68
43
29
18.4
14.3

Fig. 3.11. Southwestern blot analysis of *in vitro*-translated BAG-2 protein probed with JCV NF-1 II/III oligonucleotide.

Lanes: 1, BAG-2; 2, reticulocyte lysate; 3, luciferase protein. These proteins were resolved on a 12% SDS-PAGE gel, transferred to nitrocellulose membrane and probed with nick-translated JCV NF-1 II/III oligonucleotide. The arrow head and arrow indicate 30 and 28.5 kDa forms of BAG-2. Molecular weight markers are shown on right in kDa.


1
2
3
200
97.4
68
43
29
18.4
14.3

vitro-translated, as described in Fig. 3.7, and probed with JCV NF-1 II/III oligonucleotide also showed only two proteins of 30 and 28.5 kDa (Fig. 3.11, lane 1). The controls, the reticulocyte lysate and luciferase proteins, did not show any binding (lanes 2 and 3). Taken together, the results of immunofluorescence and Southwestern blot assays suggest that, a) BAG-2 (possibly BAG-1, see section 3.3.1) is a nuclear protein and b) two or more isoforms of BAG-2 are present in P19 cells.

3.6 Transcriptional activity of BAG-2

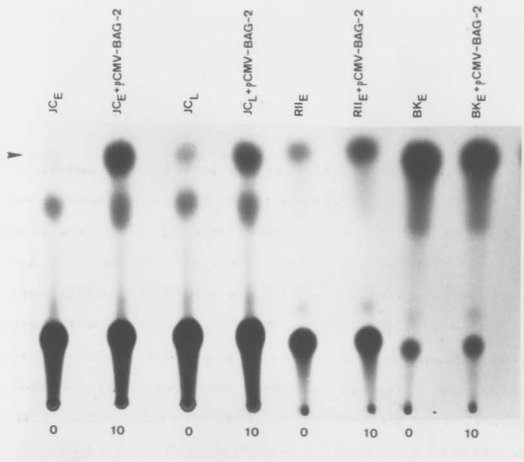
3.6.1 BAG-2 transactivation of WT JCV promoters

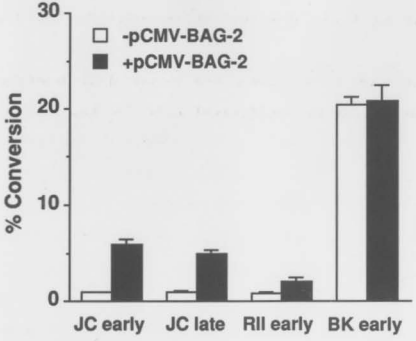
To characterize the transcriptional function of BAG-2, the cDNA was cloned into the pRc/CMV eukaryotic expression vector. The studies were done in HeLa cells, which were shown to be deficient in the brain-specific NF-1 that is important for JCV glial cell-specific expression (Tada et al., 1989). The JCV_E and JCV_L promoter-enhancer reporter plasmids were transfected either with or without pCMV/BAG-2. Transfection of JCV_E or JCV_L showed only a low level of activity (Fig. 3.12, lanes JC_E, JC_L), further indicating the glial cell-specificity of JCV. However, cotransfection of bag-2 cDNA resulted in 6-fold transactivation of JCV_E (Fig. 3.12, JC_E+pCMV-BAG-2) and 5-fold transactivation of JCV_L (Fig. 3.12, JC_L+pCMV-BAG-2). Cotransfection of the CMV vector alone had no effect (data not shown). The RII_ECAT, containing only one 98 bp repeat in the

Fig. 3.12. Transactivation of JCV promoters by BAG-2.

A. Plasmids containing the CAT reporter gene under the control of the JCV early promoter (JC_E), JCV late promoter (JC_L), one 98 bp repeat in the early orientation (RII_E) and human polyomavirus BK early promoter (BK_E) were transfected into non-glial HeLa cells. These plasmids were transfected without (lanes JC_E, JC_L, RII_E, BK_E) or with bag-2 cDNA (+pCMV-BAG-2). The arrowhead indicates the acetylated labelled CAT product. Five μg each of plasmids containing CAT, with the exception of BK with 2 μg, were transfected. The numbers below show the μg of bag-2 cDNA cotransfected with the reporter plasmids.

B. The bar graph shows the results of averages and standard deviations of 3 different experiments.


JC$_E$
JC$_E$+pCMV-BAG-2
JC$_L$
JC$_L$+pCMV-BAG-2
RII$_E$
RII$_E$+pCMV-BAG-2
BK$_E$
BK$_E$+pCMV-BAG-2
0
10
0
10
0
10
0
10


30
20
10
0
% Conversion
-pCMV-BAG-2
+pCMV-BAG-2
JC early
JC late
RII early
BK early

early orientation, was also transactivated 2.5-fold (lane RII_E+pCMV-BAG-2). As a control, BK_ECAT containing multiple NF-1 sites failed to respond to BAG-2. According to these results, the transactivation by BAG-2 was clearly shown for the JCV promoters. These results are in agreement with earlier studies (Kumar, 1994).

3.6.2 BAG-2 transactivation requirement of intact JCV NF-1 motifs

Further studies were conducted to test whether the integrity of the NF-1 sites is essential for transactivation by BAG-2. The JCV early CAT constructs containing mutations in the NF-1 motif either at regions II and III (II_{10})or I,II and III (DM_{10}) were employed. The cells used to study the effect of BAG-2 on II_{10} and DM_{10} CATs were P19 glial cells. Since, JCV itself expresses poorly in HeLa cells, it is difficult to demonstrate the effect of BAG-2 on these mutants in HeLa cell system. These mutants show a marginal activity in P19 glial cells and the effect of BAG-2 can be better appreciated in these cells.

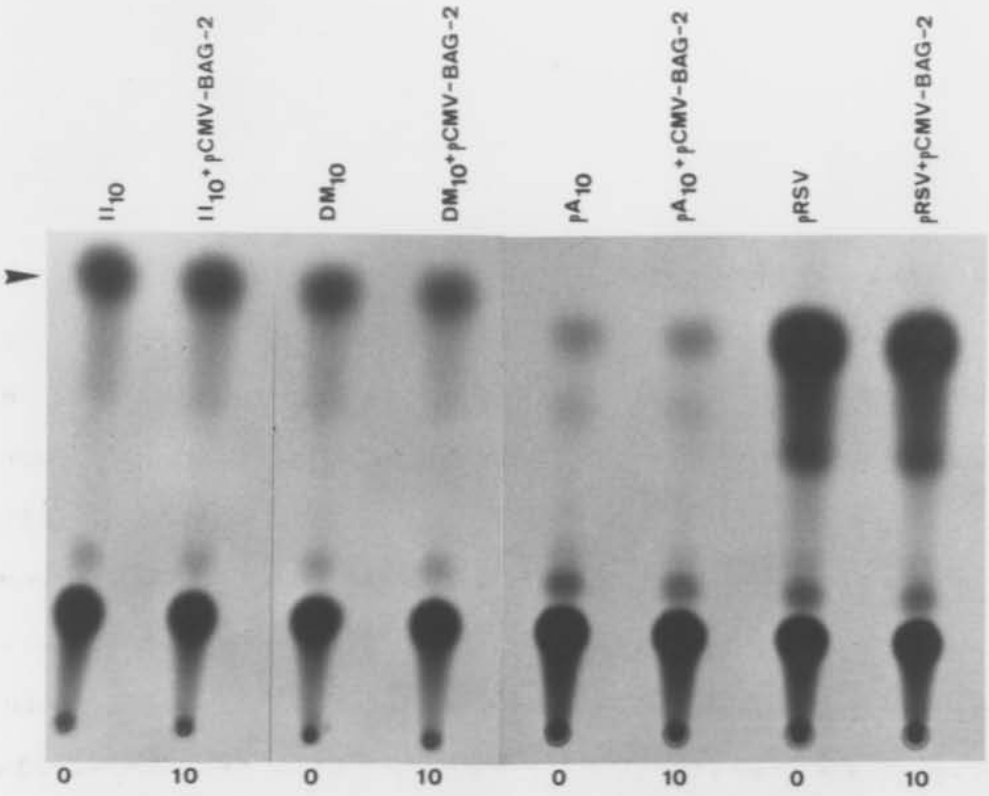
From the results in Fig. 3.13, it is clear that neither II_{10} CAT or DM_{10} CAT was responsive to BAG-2, indicating that the NF-1 motifs are essential for transactivation by BAG-2 (Fig. 3.13, lanes II_{10}+pCMV-BAG-2 and DM_{10}+pCMV-BAG-2). As a negative control, I used pA10 CAT (enhancer less SV40 promoter) that was not responsive to BAG-2. Interestingly,

Fig. 3.13. Transactivation of JCV promoters by BAG-2 requirement of intact NF-1 II/III motifs.

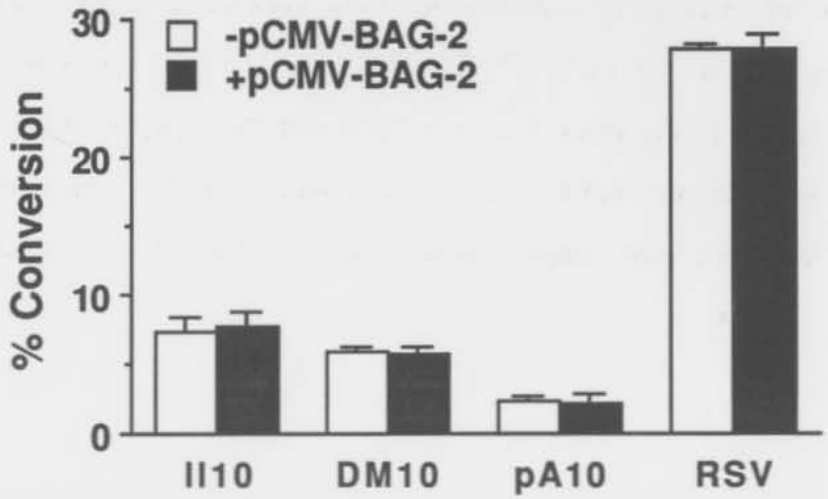
A. The CAT reporter plasmids containing JCV early promoter, bearing mutations in the NF-1 motifs in regions II and III (II_{10}), NF-1 motifs in regions I, II and III (DM_{10}), enhancer less-SV40 promoter (pA10) or Rous sarcoma virus LTR (pRSV-CAT) were transfected into P19 glial cells. These plasmids were transfected alone (lanes II_{10}, DM_{10}, pA10 and pRSV-CAT) or cotransfected with bag-2 cDNA (+pCMV-BAG-2). Two μg each of plasmids containing CAT, with the exception of pRSV-CAT with 1 μg, were transfected. The numbers below indicate the amounts of bag-2 cDNA that were transfected. The arrowhead indicates the acetylated CAT product.

B. The results for the averages and standard deviations of 3 independent experiments are shown in the bar diagram.

A


II_{10}
II_{10}+pCMV-BAG-2
DM_{10}
DM_{10}+pCMV-BAG-2
pA_{10}
pA_{10}+pCMV-BAG-2
pRSV
pRSV+pCMV-BAG-2
0
10
0
10
0
10
0
10

B


30
20
10
0
% Conversion
-pCMV-BAG-2
+pCMV-BAG-2
II10
DM10
pA10
RSV

RSV-LTR contains an NF-1 motif, TGGC TGGC that was shown to be responsive to a brain-specific transcription factor, GF-1, that is important for JCV glial cell-specific expression (Kerr and Khalili, 1991). However, I observed no such transactivation with BAG-2. Thus, my results suggested the existence of different proteins that can modulate JCV glial cell-specific expression. These results also suggest that bag-2 cDNA differs from the GF-1 cDNA isolated in Khalili's lab.

3.6.3 BAG-2 transactivation of JCV promoters in BAG-2-deficient U87 MG cells

Since Northern blot analysis showed that bag-2 mRNA is not expressed in U87 MG cells, these cells may be a good system to study the effect of BAG-2 on JCV promoters. To this end, I cotransfected JCV_E reporter plasmid and bag-2 cDNA into U87 MG cells. Cotransfection of 2.5 μg of bag-2 transactivated the JCV_E promoter by 1.25-fold. Furthermore, the transactivation appeared to be dose dependent. In other words, with an increase in concentration of cotransfected bag-2 cDNA, an increase in CAT activity was observed. A transactivation of 3-fold was seen with the maximum 15 μg of cotransfected the BAG-2 expression plasmid (Fig. 3.14).

3.6.4 Overexpression of BAG-2 exhibits squelching

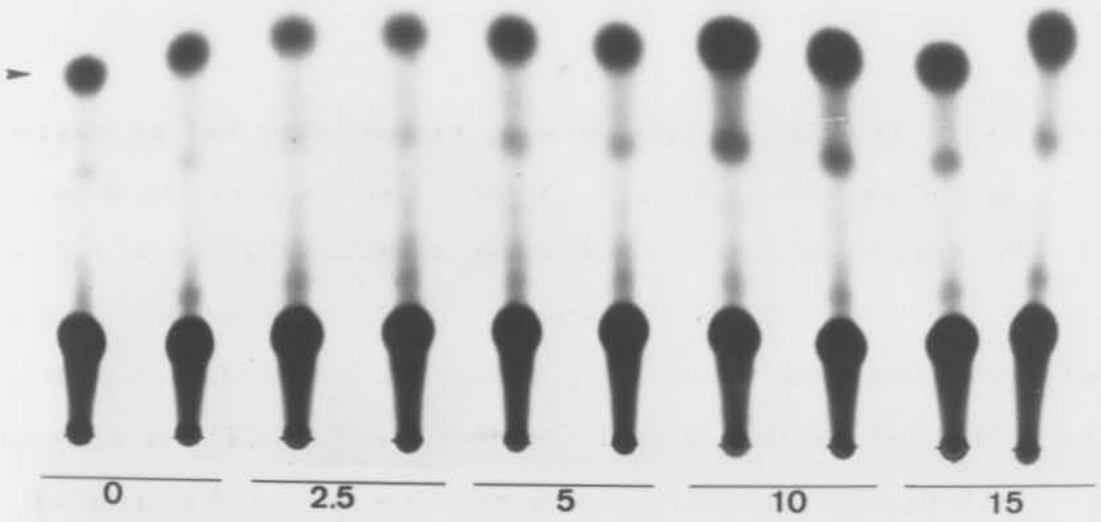
Thus far, I have examined the effect of BAG-2 on the JCV promoter in HeLa and U87 MG cells. It was intriguing to study

Fig. 3.14. Transactivation of JCV_E by BAG-2 in U87 MG cells.

A. The CAT-containing reporter plasmids under the control of JCV early promoter (JC_E) were transfected into U87 MG cells. The reporter plasmid was transfected alone or cotransfected with BAG-2 expression vector (+pCMV-BAG-2) in the number of μg indicated below. The acetylated CAT spot is indicted by an arrow head. The experiments were repeated 6 times and the results of 2 experiments are shown.

B. The bar diagram shows the averages and standard deviations of all 6 experiments.

A



B

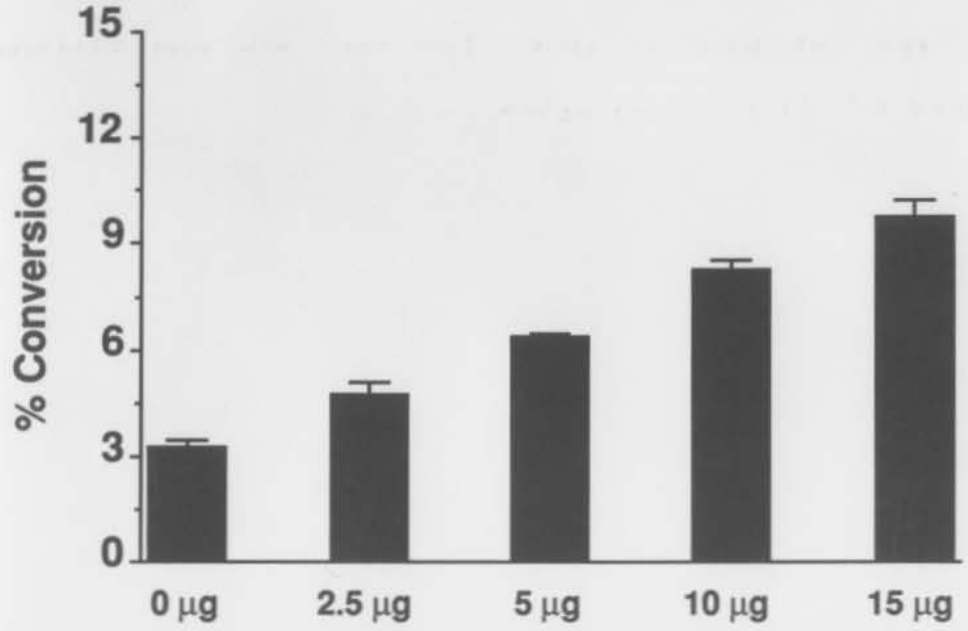
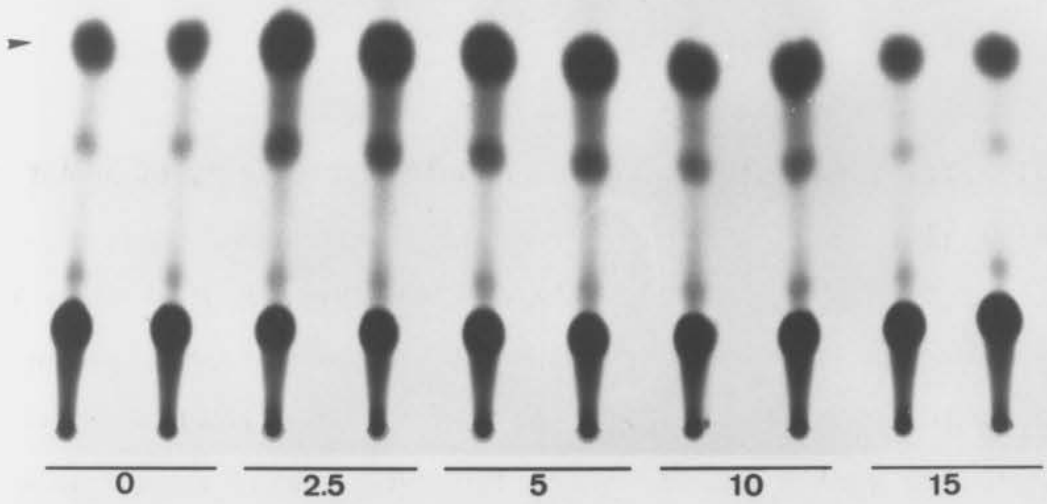


Fig. 3.15. Transactivation and squelching of JCV_E by BAG-2 in P19 glial cells.

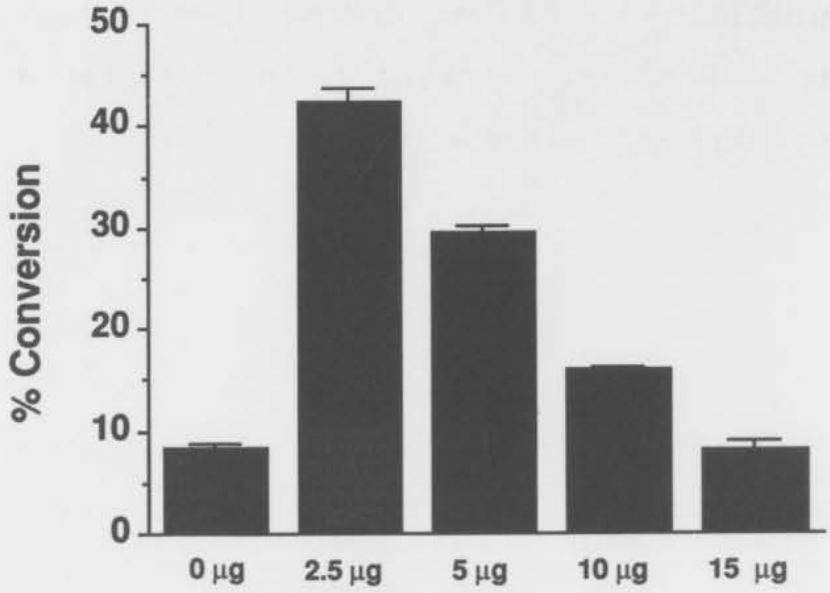
A. The JCV_E reporter plasmid was transfected into P19 glial cells. The reporter plasmid was transfected alone or cotransfected, with the μg indicated below, of the BAG-2 expression vector, pCMV-BAG-2. The experiments were repeated 6 times and the results of 2 typical experiments are shown. The arrowhead indicates the acetylated CAT product.

B. The bar diagram shows the averages and standard deviations of all the 6 experiments.

A



B



the effect of BAG-2 on JCV_E and JCV_L expression in P19 glial cells. Therefore, P19 glial cells were cotransfected with JCV_E reporter plasmid and expression plasmid for BAG-2. At 2.5 μg, BAG-2 transactivated JCV_E 5-fold, further suggesting the transcriptional function of BAG-2. Interestingly, an increase in bag-2 cDNA concentration had a negative effect above 2.5 μg. A progressive decrease in the CAT activity was observed with increasing concentration of BAG-2 (Fig. 3.15). These results are reminiscent of a phenomenon known as "squelching" (see Introduction; Berger et al., 1990; Kelleher et al., 1990; Martin et al., 1990). Similar experiments with the pCMV vector alone showed no squelching (data not shown). Squelching was similarly observed with JCV_L with an increase in the concentration of bag-2 cDNA (data not shown). By normalizing the CAT assays with the β-galactosidase expression, these results indicate a genuine squelching effect and rule out the possibility of a non-specific effect because of the overloading of the cell system or poisoning of the cellular transcriptional machinery by BAG-2. The squelching effect was also observed in in vitro transcription assays. The increase in concentration of recombinant BAG-2 resulted in the inhibition of activated, but not the basal level of, transcription from the JCV_E promoter (data not shown).

3.6.5 Bacterially produced BAG-2 stimulation of in vitro transcription from the JCV_E promoter in non-glial HeLa cells

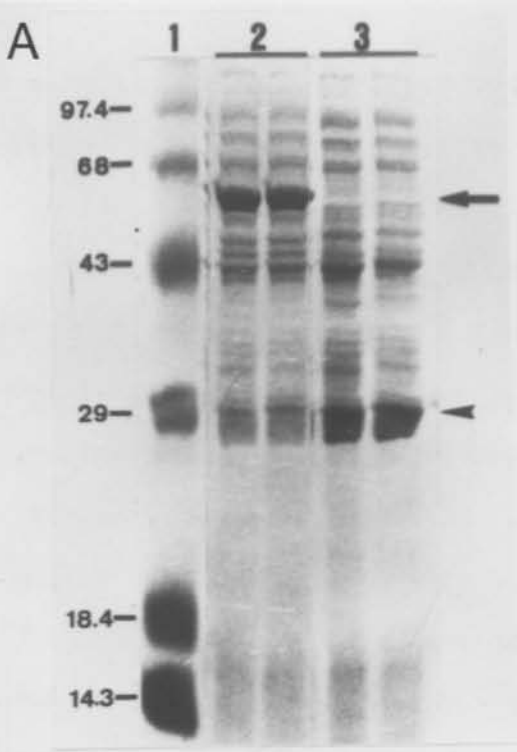
Using expression plasmids, BAG-2 clearly transactivated the JCV promoters. To further test the transcriptional activity of BAG-2, *in vitro* transcription assays were performed. To this end, a Glutathione S-transferase (GST)-BAG-2 fusion protein was made. This fusion protein was affinity-purified on a GST-Sepharose column and cleaved with thrombin to liberate the BAG-2 protein. The bacterially produced and affinity-purified GST-BAG-2 fusion protein was shown at the top in Fig. 3.16, A.

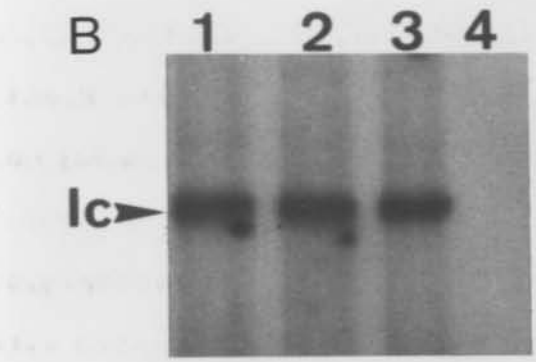
The DNA template, JC_ECAT, which gives a 160 nt nuclear run-off transcript (Tr), was used to test the transcriptional function of the purified BAG-2 protein. HeLa whole cell extract alone showed basal level activity (Fig. 3.16, B lane 2), as was also described earlier (Ahmed *et al.*, 1990a). However, the addition of 1 μg of purified BAG-2 protein resulted in a substantial 3-fold stimulation of JCV_E transcription (compare lane 2 with 3, Tr). This 3-fold stimulation was reproducible for at least 3 times. As a positive control P19 glial extract was used and showed a high amount of activity here (lane 1), as was also described previously (Kumar *et al.*, 1993). The 1 μg/mL α-amanitin control was included to demonstrate that the transcription was by RNAP II, since this concentration inhibited RNAP II (Zahler and Prescott, 1989). This concentration of α-amanitin completely blocked all transcriptional activities (lane 4). The internal control (Ic) is the adenovirus major late

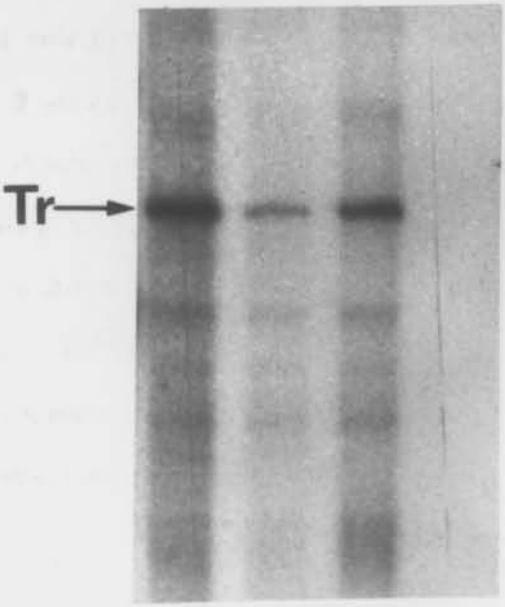
Fig. 3.16. Production of BAG-2 in bacteria and transcriptional function of purified BAG-2 for JCV_E.

A. BAG-2 in bacterial lysates. The E.coli BL21 cells containing pGEX-4T-1-BAG-2 were grown at 30^0 C in 2 liters of 2X YTA medium. The culture was grown to an A_{260} of 2.1, then induced with 0.1 mM IPTG for 2 hrs. Approximately 10 μg of each protein were resolved on a 10% SDS-PAGE gel and subjected to Coomassive blue staining. Lane 1 is molecular weight markers as indicated in the left in kDa. Lanes 2 represent the sonicates of E.coli BL21/pGEX-4T-1-BAG-2. The 60 kDa fusion protein is indicated by an arrow. Lanes 3 represent the E.coli BL21/pGEX-4T-1 sonicates. Only the GST protein (arrowhead) was detected at 30 kDa.

B. <u>In vitro</u> transcriptional function of bacterially produced BAG-2. One μg of the templates, JCV_ECAT digested with PvuII and AdMLP in pUC13 digested with EcoRI, were used. Both DNA templates were transcribed in: P19 glial extract (lane 1), HeLa extract (lane 2) and HeLa extract supplemented with 1 μg bacterially produced BAG-2 (lane 3) and P19 glial extract in the presence of 1 μg/mL of α-amanitin RNAP II inhibitor (lane 4). Run-off transcription products representing 160 nt JCV_E transcript (Tr) and 400 nt AdMLP internal control (Ic) transcript were resolved on a 5% polyacrylamide/7 M urea gel. The bands were quantified by laser densitometry with the Image Quant program from Molecular Dynamics.


A
1
2
3
97.4
68
43
29
18.4
14.3


B
1
2
3
4
Ic


Tr

promoter (AdMLP) construct, which regulates the 400 nt G-less cassette, as described (Schorpp *et al.*, 1988). Thus, the results supported the *in vivo* JCV_E expression assays (Fig. 3.12), and further indicate the *in vitro* expression for the transcriptional regulatory function of BAG-2.

3.7 Domains of BAG-2 required for DNA-binding and transactivation

3.7.1 Deletion analysis of *bag-2* cDNA

To map the regions of the BAG-2 important for transactivation and DNA-binding precisely, the cDNA was truncated at definite points with different restriction enzymes (Fig. 2.1). The truncations were verified by translating and examining the sizes of the truncated proteins by SDS-PAGE. The decrease in size of the proteins correlated with the size expected from the truncated cDNA. When a 549 nucleotide fragment was deleted from the cDNA, the resultant protein was unstable (Fig. 3.17). Since the poly(A) tail and 3'-untranslated regions were suggested to influence the efficiency of translation (Jackson and Standart, 1990) therefore, lack of poly(A) tails and 3'-untranslated regions in *bag-2* cDNAs may explain the smear-like bands observed for the mutant BAG-2 proteins.

3.7.2 The BAG-2-specific C-terminus is essential for transactivation

Fig. 3.17. SDS-PAGE analysis of *in vitro*-synthesized truncated BAG-2 proteins.

The *bag-2* cDNA in pBSIIKS$^+$ was truncated at distinct sites with restriction enzymes, as indicated in Fig. 2.1. The ^{35}S-labeled proteins were synthesized using 1 μg of *bag-2* cDNA as template and a kit from Promega. The expected sizes of the truncated proteins are: Δ244, 27 kDa; Δ371, 22 kDa; Δ477, 18 kDa; Δ511, 17 kDa; Δ602, 14 kDa; Δ715, 9 kDa. The truncated BAG-2 proteins are indicated by arrowheads and the wild-type protein is indicated by an arrow.

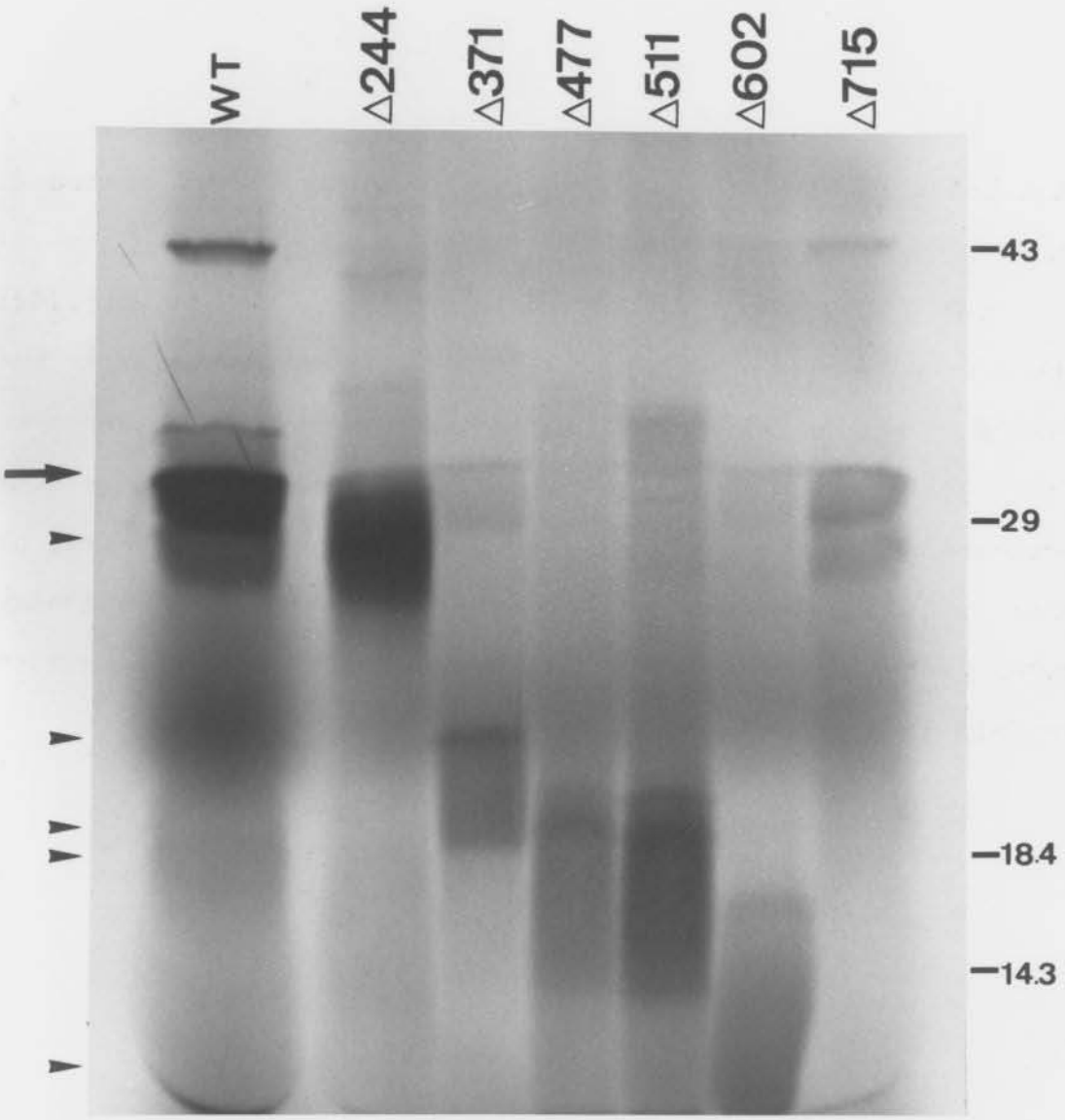

WT
Δ244
Δ371
Δ477
Δ511
Δ602
Δ715
43
29
18.4
14.3

Fig. 3.18. Domains of BAG-2 required for transactivation.

A. The truncated bag-2 cDNAs were described in Fig. 2.1, cloned into the pCMV vector and cotransfected with 10 μg CAT reporter plasmid regulated by the JCV_E (JC_E) as indicated above. The non-glial HeLa cell system was used. The arrow head indicates the acetylated CAT product.

B. The results for the averages and standard deviations of 3 experiments are shown.

A

JC$_E$
JC$_E$+pCMV-BAG-2
JC$_E$+pCMV-Δ244
JC$_E$+pCMV-Δ371
JC$_E$+pCMV-Δ511
JC$_E$+pCMV-Δ602

B

% Conversion
0
2
4
6
8
JCV early
JCV early+WT
Δ244
Δ371
Δ511
Δ602

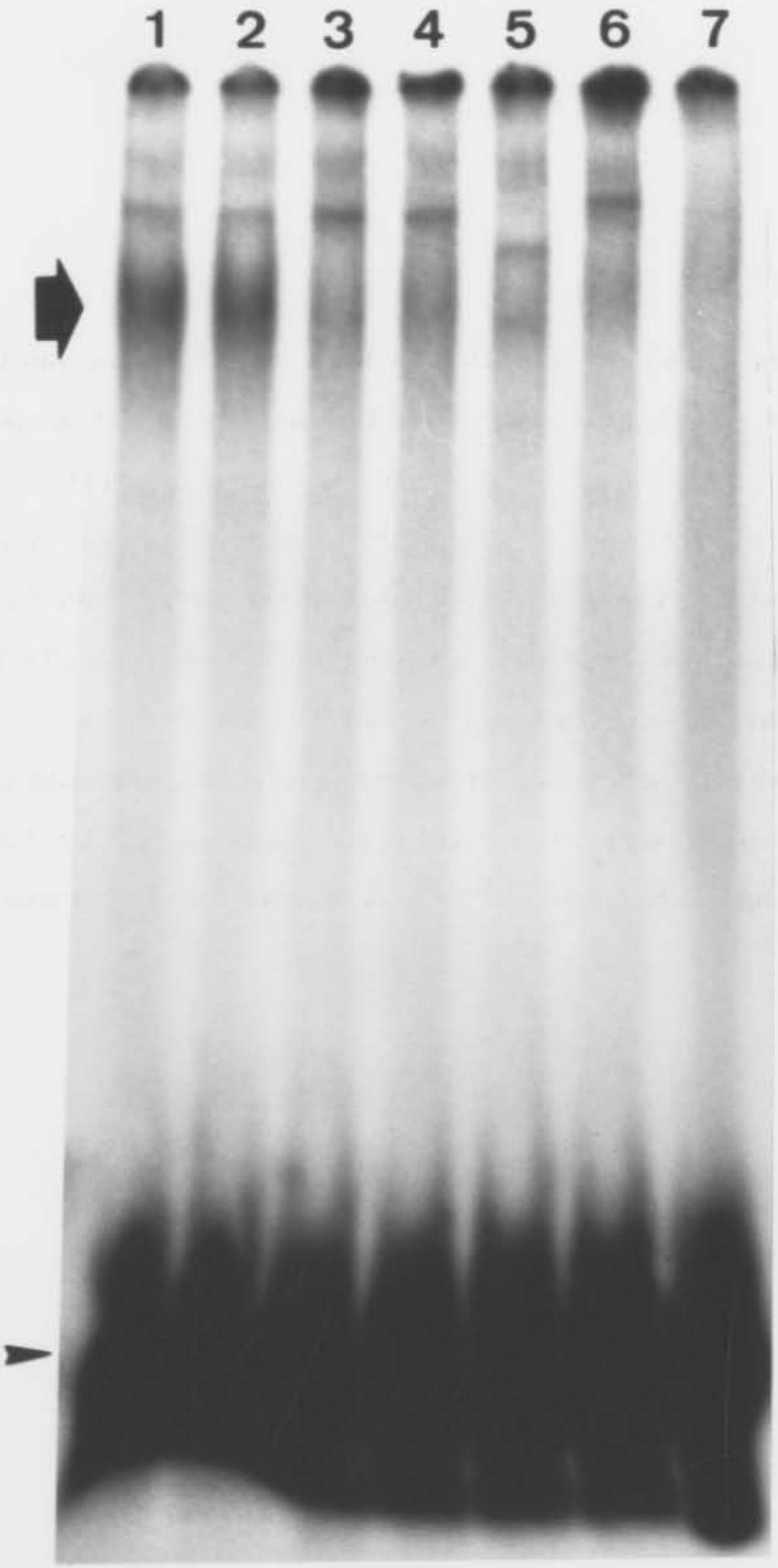
The truncated cDNAs were cloned into the pRc/CMV vector (NOTE: the pRc/CMV vector provides the poly (A) tail and the 3'-untranslated region, so the efficiency of translation will not be compromised here) and cotransfected with the JCV_E reporter plasmid. Deletion of the C-terminus resulted in a dramatic decrease in transcriptional activation by BAG-2 (Fig. 3.18). Only 1.9-fold transactivation was observed. The result was intriguing, since only the C-terminus distinguished between BAG-1 and BAG-2. These results suggest that BAG-2 is transcriptionally active. These results indicated that the transcription activation property of BAG-2 is conferred by the C-terminus. Thus, these results indicated that BAG-2 is a novel, unique transcription factor. Other deletions, which remove the central portion of the cDNA, also had an effect on transactivation. This effect may be due to lack of DNA-binding property sine this region is essential for DNA-binding (see below). Finally, the deletion of a large proportion of cDNA abrogated the transactivation property of BAG-2. These results clearly demonstrated that BAG-2 is essential for JCV transcription. Furthermore, these results rule out any non-specific effect of pCMV on the JCV_E promoter.

3.7.3 DNA-binding activity of the central coiled-coil region of BAG-2

The truncated cDNAs were <u>in vitro</u>-translated to test the DNA-binding activity. Removal of the C-terminus had no effect

Fig. 3.19. *In vitro* binding to NF-1 II/III oligonucleotide of truncated BAG-2 proteins in gel mobility shift assay.

The truncated *bag-2* cDNAs (Fig. 2.1) were non-radioactively translated *in vitro*. Approximately 4 μL of each *in vitro*-translated protein and nick-translated NF-1 II/III oligonucleotide were used in binding assays. Lanes: 1, WT BAG-2 protein; 2, Δ244 BAG-2; 3, Δ371 BAG-2; 4, Δ511 BAG-2; 5, Δ602 BAG-2; 6, Δ715 BAG-2; 7, free oligonucleotide probe with no protein. Broad arrow indicates low-mobility DNA-protein complex and the arrowhead indicates protein-unbound free probe.


1
2
3
4
5
6
7

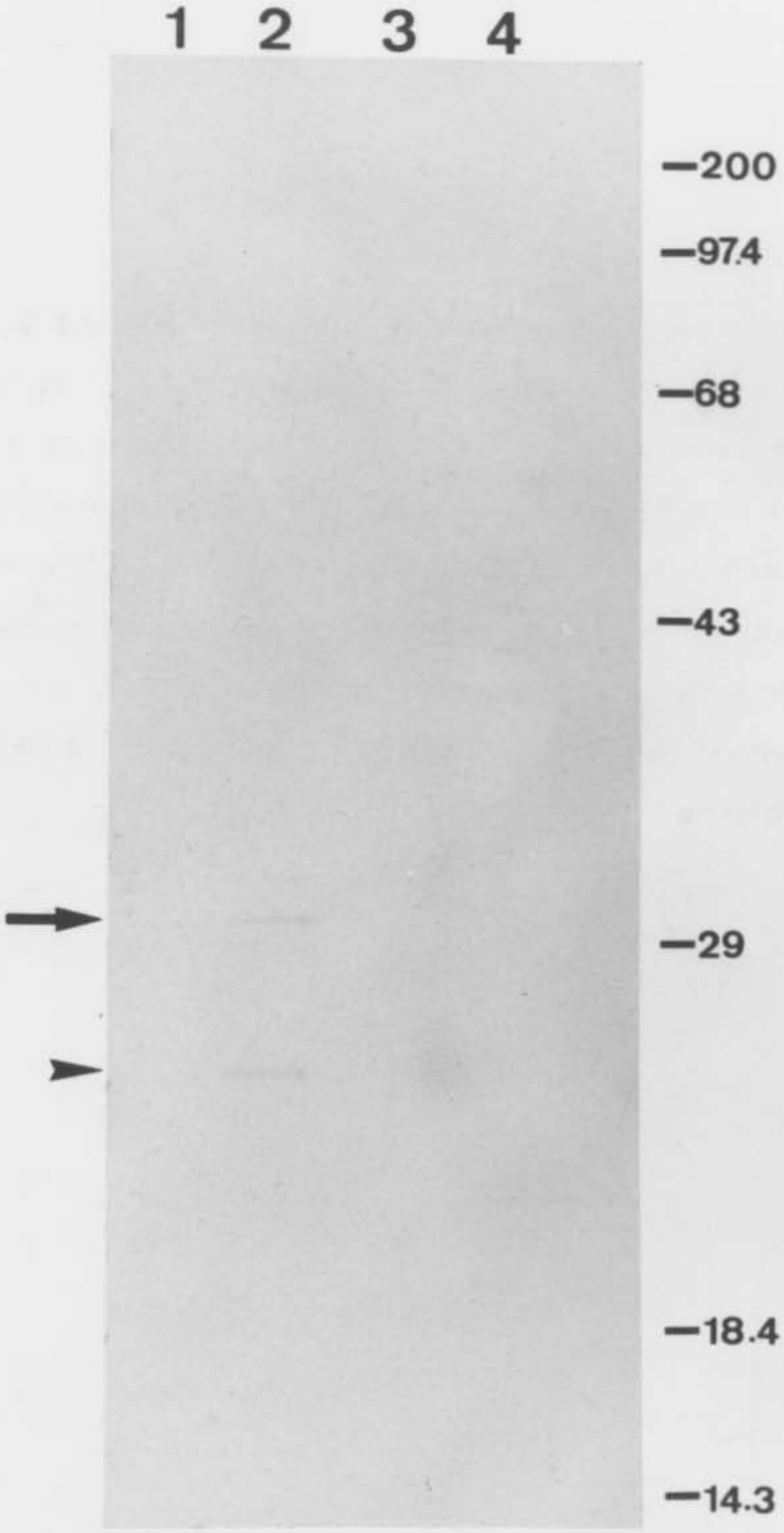
on the DNA-binding activity (Fig. 3.19, lane 2). However, removal of the central coiled-coil region had a dramatic effect on DNA-binding, suggesting that this region was essential for DNA-binding (lanes 4,5 and 6). These results were reproducible for three times. Taken together, these results suggested that the transactivation domain is in C-terminus and the DNA-binding domain is in the central coiled-coil region. Computer analysis of these two regions failed to reveal classical activation or DNA-binding domains. Since the activation domains of most eukaryotic transcription factors are poorly studied (Mitchell and Tjian, 1989; Ptashne and Gann, 1988), the BAG-2 C-terminus may define a novel transactivation domain. On the other hand the central region has several positively charged amino acids. These positive charges may confer DNA-binding property, since positively charged amino acids were shown to be essential for DNA-binding of CTF/NF-1 (Mermod *et al.*, 1989).

3.8 BAG-2 interacts with Bcl-2

My studies revealed that BAG-2 is a novel transcription factor. I was interested in examining whether or not BAG-2 interacts with Bcl-2, because of the fact that BAG-1 does (Takayama *et al.*, 1995). For this purpose, the Far-Western blot technique was employed. Clearly, radiolabelled *in vitro*-translated BAG-2 protein specifically interacted with the immobilized Bcl-2 (Fig. 3.20, lane 2, arrowhead), but not with

Fig. 3.20. *In vitro* interaction between BAG-2 and Bcl-2.

A Far-Western blot is shown. Lanes: 1, BSA; 2, *in vitro*-translated Bcl-2; 3, reticulocyte lysate only; 4, *in vitro*-translated luciferase. Proteins were resolved on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. The immobilized proteins were probed with radiolabelled BAG-2 protein. Molecular weight markers are shown on the right in kDa. The arrow indicates 30 kDa and arrowhead indicates 26 kDa forms of Bcl-2.


1
2
3
4
200
97.4
68
43
29
18.4
14.3

the reticulocyte lysate (lane 3), *in vitro*-translated luciferase (lane 4) or BSA (lane 1). The arrowhead indicates the 26 kDa Bcl-2 protein and the arrow indicates the 30 kDa form of Bcl-2 which may be arising from the posttranslational modification of Bcl-2 in the *in vitro* translation reaction. These results are not surprising since the BAG-1's ubiquitin-like domain was speculated to be essential for interaction with Bcl-2 (Takayama *et al.*, 1995) and BAG-2 might have interacted with Bcl-2 via the same domain. The interaction between BAG-2 and Bcl-2 was consistently observed in three independent experiments. However, this result may help to explain the puzzling latency of the JCV in the kidney, and also in B cells (see Discussion).

3.9 Effect of BAG-2 on murine p53 promoter

3.9.1 Transcriptional repression of mouse p53 promoter by BAG-2

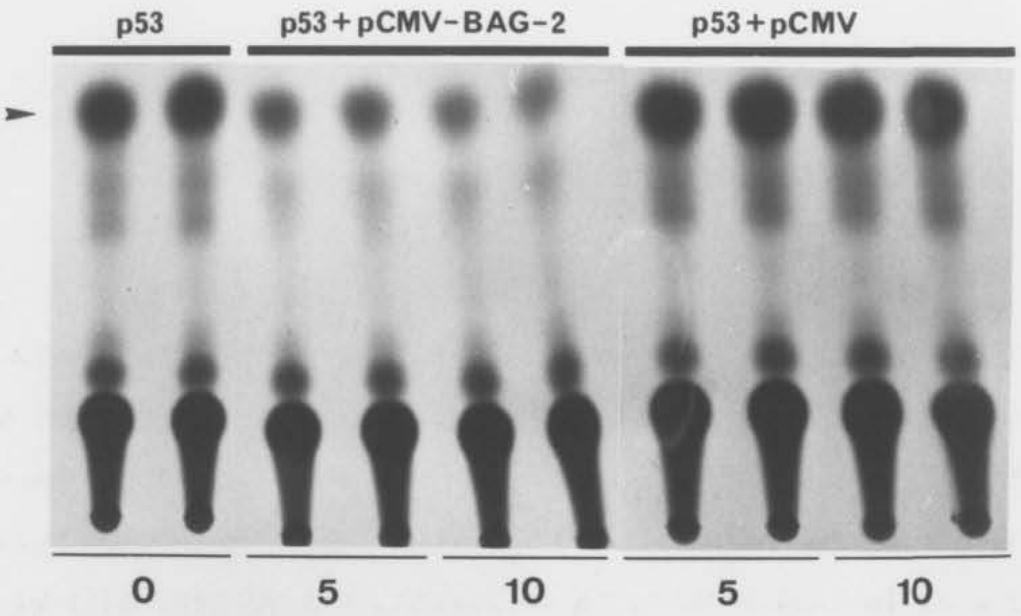
As discussed in the Introduction, p53 was shown to negatively regulate expression of *bcl-2* by interacting with a negative regulatory element (NRE) in the *bcl-2* gene (Young and Korsmeyer, 1993; Miyashita and Reed, 1994b). Interestingly, p53 was recently shown to transactivate the promoter of *bax*, the antagonist of Bcl-2 and inducer of apoptosis (Miyashita and Reed, 1995). Thus, p53 appears to induce apoptosis by decreasing Bcl-2 and increasing Bax levels. This intricate

Fig. 3.21. Effect of BAG-2 on murine p53 promoter.

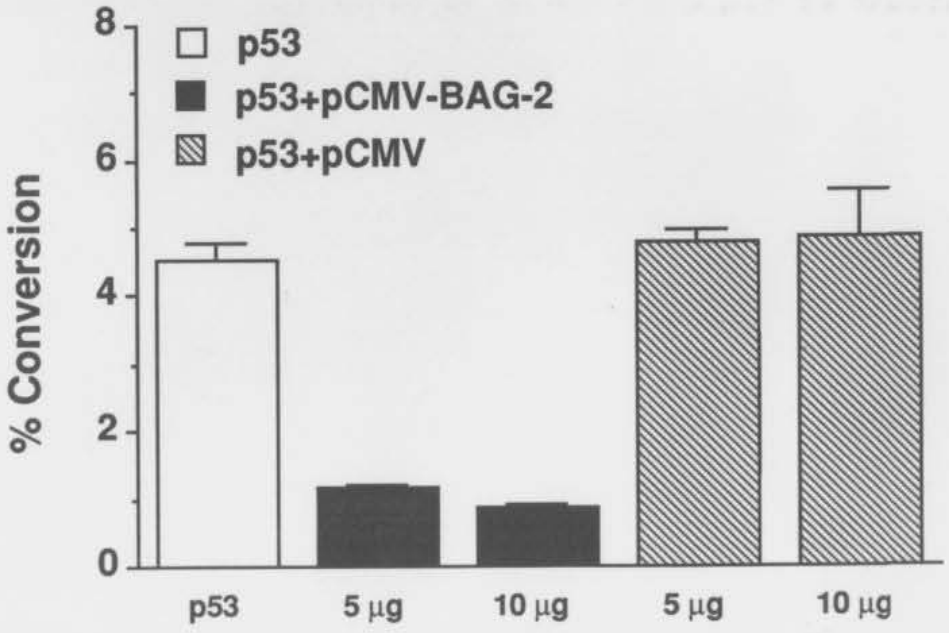
A. Two μg CAT reporter plasmid regulated by the mouse p53 promoter was transfected into P19 glial cells either with or without the indicated μg shown below of BAG-2 expression plasmid (+pCMV-BAG-2) or with the pCMV vector. The acetylated CAT spot is indicated by an arrowhead. The results of 2 of the 8 experiments are shown.

B. A bar diagram gives the averages and standard deviations of the 8 different experiments.

A


p53
p53 + pCMV-BAG-2
p53 + pCMV
0
5
10
5
10

B


% Conversion
8
6
4
2
0
p53
p53+pCMV-BAG-2
p53+pCMV
p53
5 µg
10 µg
5 µg
10 µg

control may be necessary to ensure the careful regulation of cell proliferation (Hockenbery, 1995). Since Bcl-2 inhibits apoptosis, Bcl-2 may inhibit p53 via an uncharacterized mechanism. BAG-1 is known to be an abettor of Bcl-2 in the inhibition of apoptosis. Since my studies discovered that BAG-2 has transcriptional properties, BAG-2 may have some influence on the expression of p53. The rationale is that, since BAG-2 binds to the JCV NF-1 II/III motif, BAG-2 may interact with p53 promoter via the NF-1 motif. The NF-1 motif is present in the p53 promoter, but has not been functionally characterized (Ginsberg et al., 1990). The rationale for using the p53 promoter of mouse encompasses two reasons. First, BAG-2 was cloned from mouse embryonal carcinoma cells. Second, it is appropriate to study the relationship between BAG-2 and mouse p53 promoter in a mouse cell system.

Therefore, the mouse p53 promoter regulating the CAT reporter plasmid was transfected into P19 glial cells either with or without pCMV-BAG-2. The p53CAT alone showed a significant activity. Impressively, cotransfection with 5 and 10 μg bag-2 cDNA resulted in a 3-fold and 4-fold repression, respectively (Fig. 3.21). The same concentrations of the pCMV vector had no effect. Thus, the results indicated that BAG-2 down-regulates the p53 promoter.

CHAPTER 4

DISCUSSION

These studies were done with the objective of characterizing the cDNA cloned from a P19 glial cell cDNA library using JCV NF-1 II/III motif as a probe (Kumar, 1994). Sequence analysis of the cDNA revealed a novel protein that showed 85% homology to an important, recently reported, Bcl-2 interacting protein called BAG-1 (Takayama et al., 1995). However, the C-terminus had no homology to BAG-1 (Fig. 3.2). Hence I named the cDNA bag-2. BAG-2 is important for glial cell-specific expression of JCV (Fig. 3.12 and 3.13).

JCV is a strict human neurotropic virus, and the tissue-tropism was shown to be conferred by the two 98 bp repeats of the regulatory region (Kenney et al., 1984; Nakshatri et al., 1990a; Tada et al., 1989). JCV remains latent in kidneys, B cells and peripheral blood leucocytes after the initial infection (Houff et al., 1988; Dorries et al., 1994). The latent infection becomes activated during immunosuppression brought by myriad of causes (reviewed in Frisque and White, 1992). One reasonable speculation to explain the occurrence of viral reactivation during immunosuppression is that the cytokine-inducible NF-κB and GBPi transcription factors activate JCV_E gene expression (Ranganathan and Khalili, 1993; Raj and Khalili, 1994). Thus, a change in the cytokine profile

would alter JCV gene expression and lead to productive infection and haematogenous spread of JCV to the brain and other organs (Ault and Stoner, 1994; Atwood et al., 1995). Although JCV is transported to all the organs by haematogenous spread during reactivated infection, JCV DNA replication and expression occur only in the brain. Many studies have demonstrated the importance of brain-specific transcription factors, like NF-1, AP-1, YB-1, Pur α, NF-κB and domain D interacting protein, in JCV expression (Khalili et al., 1988; Ahmed et al., 1990b; Amemiya et al., 1992; Ranganathan and Khalili, 1993; Kerr et al., 1994; Chen et al., 1995 a and b). Virtually none of these studies showed conclusively which interaction is important for glial cell-specific expression of JCV in vivo. More recently, the JCV core promoter containing only the TATA box and the adjacent poly A region was shown to be glial-specific (Krebs et al., 1995). This argues against the role of other transcription factors in glial cell-specific expression of JCV. The above study is analogous with a previous study, in which TFIID from brain, but not from liver, was shown to confer the brain-specific expression of myelin basic protein (MBP) (Tamura et al., 1990a). This suggests that different tissues contain different TFIID complexes that can regulate JCV gene expression differentially. A corollary to this is that such a brain-specific TFIID governs the brain-specific expression of JCV. This also suggests the functional redundancy by the two TATA boxes of the 98 bp repeat in the

JCV regulatory region for glial cell-specific expression.

Studies from our laboratory using a uniquely defined system-P19 embryonal carcinoma cells-attempted to unravel the molecular mechanism of JCV glial cell-specific expression. The P19 undifferentiated cells can be differentiated with RA into neuronal cells and with DMSO into muscle cells (McBurney, 1993). Thus, with one cell system, the tissue-specific expression of JCV can be studied. Therefore, JCV was shown to express only in P19 glial but not in UD or P19 muscle cells (Nakshatri et al., 1990a). The same study also identified three NF-1 motifs in the JCV regulatory region by DNase I footprinting with nuclear extracts from P19 glial cells. Subsequently, site-directed mutagenesis was used to show the importance of the NF-1 motifs in the 98 bp repeats for strict glial cell-specific expression of JCV_E (Kumar et al., 1993). A cDNA was cloned from P19 glial cell cDNA library using JCV NF-1 II/III oligonucleotide (Kumar, 1994).

In my studies, sequence analysis of this cDNA revealed a large ORF of 687 nucleotides potentially coding for a 30 kDa protein. A homology search lead to a surprising finding. The protein was perfectly identical (85% identity), except for the C-terminus (a.a 195 to a.a 229) (Fig. 3.2), to a novel and interesting Bcl-2 interacting protein called BAG-1 (Takayama et al., 1995). My finding was surprising, considering that the JCV NF-1 motif interacted with a Bcl-2-interacting protein. Analysis of the bag-2 cDNA sequence identified 3 ATGs at the

5' end of the cDNA. The first ATG is in a strong favourable context for translation initiation, since 7 of 12 nucleotides upstream of the first ATG match the consensus sequence observed in the majority of eukaryotic mRNAs (Kozak, 1987) (Fig. 3.1). However, translation from second and third ATGs can not be excluded, since leaky scanning (bypassing by the 40S ribosomal subunits of the first ATG and initiating protein synthesis at downstream ATGs) was shown to produce different isoforms of proteins from a single species of mRNA. Examples for such a leaky scanning though the first ATG is in favourable context, are cellular genes such as creatine kinase in chicken brain, N-myc in human tumor cell lines (Makela et al., 1989; Soldati et al., 1990) and viral genes of influenza virus B (Williams and Lamb, 1989).

ATG-burdened leader sequences are common among proto-oncogenes, transcription factors and receptor proteins. Such ATG-burdened leader sequences impair translation efficiency (Kozak, 1989). Thus, mRNAs encoding critical regulatory elements are intended to be translated poorly, thereby critically regulating the cellular homeostasis. One of the important proto-oncogenes is bcl-2. Bcl-2 mRNA was shown to have ATG-burdened leader sequences and was shown to be translated inefficiently (Tsujimoto and Croce, 1986). BAG-1 was shown by gene transfer studies to inhibit apoptosis, either alone or by interacting with Bcl-2 (Takayama et al., 1995). Thus, bag-1 may represent a new class of proto-

oncogenes. Therefore, the presence of multiple ATGs in 5' leader sequences is not surprising. However, recent results suggest that eukaryotic translation occurs from the first ATG, despite the presence of a second, nearby ATG. That is, the first ATG is preferred for translation initiation (Kozak, 1995). Thus, it appears that the first ATG in bag-2 initiates translation. However, the next 2 ATGs are also in a favourable context for translation initiation. Therefore, any of the ATGs may be utilized.

In vitro-translation of the bag-2 cDNA yielded two proteins of 30 and 28.5 kDa (Fig. 3.7). The 30 kDa protein apparently arises from first ATG and the 28.5 kDa protein most likely arises from the third ATG, based on the respective molecular weight predictions from the ORF sequences. Recent studies in eukaryotes suggest that, if two 5' end ATGs are closely located, the first ATG would be preferred for the initiation of translation (Kozak, 1995). Consistently, the 30 kDa protein is more abundant than the 28.5 kDa species. Other examples, in which two protein isoforms were shown to arise from a single species of mRNA were creatine kinase and N-myc (Soldate et al., 1990; Makela et al., 1989). Though these two protein isoforms were shown to have an identical function, the biological significance for generating such protein isoforms is unknown. Viruses also are known to use a similar translational ploy to generate protein isoforms. For example, a second ATG adjacent to the first ATG was shown to allow

leaky scanning to produce NB and NA glycoproteins in influenza B virus (Williams and Lamb, 1989).

The poly(A) signal in bag-2 cDNA (AATGAAA) deviates from the consensus poly(A) tail, AATAAA. However, the G at 4th position was observed in 15% of mRNAs in vertebrates. Further, mutations in the poly(A) signal impair cleavage and polyadenylation, the site is not functionally the rate-limiting step in gene expression (Sheets et al., 1990).

The cDNA cloned from our P19 glial cell cDNA library was discovered to code for a Bcl-2-interacting protein, BAG-2. Further, BAG-1 was suggested to be ubiquitous, since it was cloned from a mouse embryonic cDNA library (Takayama et al., 1995). Therefore, it was essential to test the tissue-specific distribution of the homologous BAG-2 to address the glial cell-specificity of JCV. The Northern blot analysis detected bag-2 in UD, P19 muscle and P19 glial cells, but not in U87 MG or HeLa cells (Fig. 3.5). U87 MG cells support JCV expression (Fig. 3.14) although, BAG-2 is absent in these cells. This may indicate that BAG-2 is not the only transcription factor conferring the glial cell-specificity to JCV (see section 1.2.3). Since BAG-1 and BAG-2 differ at their C-termini, the BAG-2 C-terminus may somehow confer tissue-specific function. The detection of the BAG-2-specific C-terminus in UD, P19 muscle and P19 glial cells, but not in HeLa cells, further complicated the possible mechanism for the glial cell-specificity of JCV.

JCV is expressed in P19 glial cells, but not in UD or P19 muscle cells (Nakshatri et al., 1990a). My studies revealed the importance of the C-terminus of BAG-2 for transcriptional activation (Fig. 3.18) and the central coiled-coil region for DNA-binding (Fig. 3.19). These results therefore suggest that the C-terminus is important for BAG-2 transactivation, but the C-terminus is specific to the P19 cells. Possible explanations for a BAG-2 role in glial cell-specific expression of JCV are: 1) Specific nuclear translocation; 2) BAG-2 may be degraded specifically in non-glial cells, as supported by the presence of ubiquitin-like domain (Fig. 3.3 B); 3) BAG-2 may be specifically translated in glial cells, as revealed by the Southwestern blot and immunofluorescence results; 4) The other interacting transcription factors in glial cells may modulate the effect of BAG-2; 5) A negative regulatory element (NRE) is found immediately adjacent to the NF-1 sites in the JCV regulatory region (Fig. 1.2). Further, the factors that bind to NRE were shown to negatively regulate JCV expression in non-glial cells (Tada et al., 1989; Tada et al., 1990; Sharma and Kumar, 1991). In glial cells probably, BAG-2 occupies the NF-1 motifs preventing the interaction of negative regulators with NRE thus, allowing glial cell-specific expression of JCV. Thus, JCV glial cell-specific expression may be controlled by cis-elements that act positively in glial cells and negatively in non-glial cells. Furthermore, the trans-acting factors from non-glial cells that bind to the JCV regulatory region were

revealed to be different, by *in vitro* gel mobility shift assays (Khalili *et al.*, 1988; Kumar *et al.*, 1993). Non-glial cells also were demonstrated not to have the brain-specific NF-1 that is important for JCV expression (Tada *et al.*, 1989; Tamura *et al.*, 1990b); and 6) Alternatively, a short leader sequence with not many ATGs in the 5'-leader sequence may be important. The *bag-2* cDNA contains three ATGs and such leader sequences may be translated more efficiently in P19 glial cells. The higher levels of protein may be due to more efficient translation in glial cells. In contrast, the 5' leader sequences with many ATGs may be translated poorly in non-glial cells. The possible poor translation is suggested by the faint band of BAG-2 observed in Southwestern blot analysis with NE from P19 muscle cells and UD cells. However, the same amount of nuclear extract revealed a strong band for P19 glial cells (Fig. 3.10). Also in immunofluorescence assays there was clearly less BAG-2 protein in UD than P19 glial cells (Fig. 3.4, 2B). The Southwestern blot quantitatively showed that the expression was much greater in P19 glial cells than in P19 muscle cells (Fig. 3.10) and UD cells (data not shown). These results were completely consistent with the Northern blot analysis, in which the same amounts of mRNA were detected in all P19 cells but the protein level varied (Fig. 3.5). This indicates that translational control is critical for the expression of BAG-2 in P19 cells. The other possibility is that external signals such as, RA may influence the

translation efficiency of BAG-2. For example, translation of the human HOX-5.1 gene appeared to be controlled by the external signal, RA. The 5' leader sequences of HOX-5.1 having fewer ATGs were added in response to RA (Cianette et al., 1990). However, having many ATGs in the 5'-leader sequences may severely impair the translation efficiency in UD and P19 muscle cells.

Preliminary work with bag-2 cDNA suggested that it transactivates both JCV promoters equally (Kumar, 1994). However, whether such transactivation required the integrity of the NF-1 motifs in the JCV regulatory region was unclear. In my studies, cotransfection experiments showed that BAG-2 transactivated JCV_E 6-fold and JCV_L 5-fold (Fig. 3.12). Interestingly, the RII_E CAT containing one 98 bp repeat in an early orientation was also transactivated 2.5-fold (Fig. 3.12). Since the JCV regulatory region is bidirectional in function, these results suggest that one repeat is sufficient for BAG-2 transactivation. However, no such transactivation was observed with JCV containing mutations in both NF-1 motifs (Fig. 3.13). Thus, these results suggest that transactivation by BAG-2 is specifically dependent on the integrity of the NF-1 motifs. Moreover, the transactivation is very specific to JCV, since another human polyomavirus, BKV, containing multiple NF-1 sites in its regulatory region was not responsive to BAG-2 (Fig. 3.12). The negative control pA10CAT was not responsive to BAG-2. Furthermore, recombinant BAG-2

protein specifically stimulated transcription from the JCV_E (Fig. 3.16). Taken together, these results suggest that BAG-2 transactivates specifically the gene expression of JCV and this requires the integrity of the NF-1 motifs.

Previous work from Khalili's lab purified a factor from calf brain that interacts with the JCV NF-1 motif and was shown to transactivate JCV_E (Ahmed et al., 1990a). Later work from the same lab cloned a cDNA by screening a human brain cDNA library with a JCV NF-1 probe. This cDNA (GF-1 cDNA) was shown to transactivate JCV_L (Kerr and Khalili, 1991). Though, the same probe was used in both the systems, the results were contradictory and highly inconclusive. However, the bag-2 cDNA characterized here transactivated both JCV promoters equally. Interestingly, GF-1 cDNA was shown also to transactivate RSV-LTR containing NF-1 motif (Kerr and Khalili, 1991). On the other hand, RSV-LTR was not responsive to BAG-2 (Fig. 3.13). Thus, these results clearly demonstrated the existence of different brain-specific factors in different cells that modulated JCV expression differentially. Further, these results suggest that the BAG-2 cDNA is entirely different from the cDNA cloned by Khalili's lab, based on functional as well as sequence data.

Yet another interesting finding with Southwestern blots was the detection of BAG-2 in NEs from P19 glial cells and much lower levels from HeLa and P19 muscle cells (Fig. 3.10). This indicated that BAG-2 was translocated into the nucleus.

The immunofluorescence data displayed the nuclear localization of the BAG-2 only in P19 glial cells, not in UD cells (Fig. 3.4, 2B). Only scattered nuclear staining was observed in P19 muscle and HeLa cells (data not shown). The combined results suggest that BAG-2 is translated efficiently and transported into nucleus only in P19 glial cells. The limited nuclear localization in UD, P19 muscle and HeLa cells is consistent with JCV expression levels being much higher in P19 glial cells than UD and P19 muscle cells (Kumar et al., 1993). Since the immunofluorescence analysis used an antibody that cross-reacts with BAG-1 N-terminus, it is possible that both BAG-1 and BAG-2 or BAG-2 alone can be translocated into nucleus. This can be further tested by generating the BAG-2 specific antibody. Since the BAG-2 specific C-terminus is essential for transactivation (Fig. 3.18), only BAG-2 can transactivate JCV promoters, but not BAG-1 despite its nuclear localization. Recent results suggest that BAG-1 exists in the cell as a multicomplex protein associating with various cellular proteins such as TFII D. Furthermore, these results have also indicated the differential subcellular localization of BAG-1 (Gehring, Pers. Comm). Thus, BAG-1 is not only a multicomplex protein but also a multifunctional protein (John Reed, Pers. Comm). Hence, it is also possible that BAG-2 may have similar functions partly because of its differential subcellular localization.

My results may partially explain the latency of JCV in

kidneys and B cells. Though the BAG-2 was expressed in UD, P19 glial and P19 muscle cells, the reason for the protein remaining in the cytoplasm may be the existence of a deficient nuclear transport system or the association of BAG-2 with Bcl-2 or other factors. Thus, a cytoplasmic BAG-2 protein would protect the cells from apoptosis during viral infection, facilitating persistence and negatively regulating p53 expression. Both would suppress p53-induced apoptosis. If immunosuppression occurs, viral reactivation and transportation to the brain could follow. Possibly, in the brain, the BAG-2 protein would be released from its association with Bcl-2 or other proteins. However, this is analogous with the transcription factor, NF-κB. Although ubiquitously expressed, NF-κB is active only in B cells upon its nuclear translocation. In the cytoplasm NF-κB is inactive because the IκB inhibitor interacts with the 110 kDa precursor of the p50 subunit of NF-κB and masks its NLS (Beg et al., 1992). Cytokines disrupt the NF-κB/IκB complex. Thus, NF-κB becomes translocated into the nucleus (Baeuerle and Baltimore, 1988). My demonstration of the BAG-2/Bcl-2 interaction (Fig. 3.20) may indicate a similar putative mechanism described above.

Since alternative splicing did not introduce an NLS for BAG-2 (Fig. 3.3, B) the other mechanisms for translocation may be important. In the brain, only lytic infection of the oligodendrocytes is observed. Hence, the transport of BAG-2

Fig. 4.1. Model for regulation of expression of JCV by BAG-2.

A. The putative role of BAG-1/2 in JCV latency in kidney and B cells.

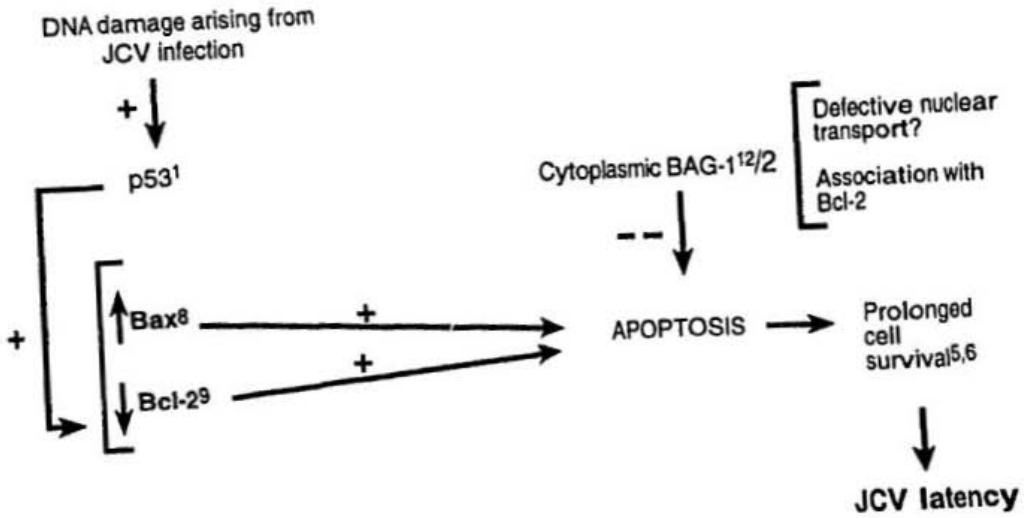
B. The putative involvement of BAG-2 in JCV expression in brain cells.

See the text for abbreviations and details. This model is not an exhaustive compilation of literature. However, the references cited are:

1, Ariza et al., 1994; 2, Ault and Stoner, 1994; 3, Berke, 1994 and 1995; 4, Heusel et al., 1994; 5, Hockenbery, 1995; 6, Korsmeyer, 1995; 7, Major et al., 1992; 8, Miyashita and Reed, 1995; 9, Miyashita et al., 1994a; 10, Raj and Khalili, 1994; 11, Ranganathan and Khalili, 1993; 12, Takayama et al., 1995;

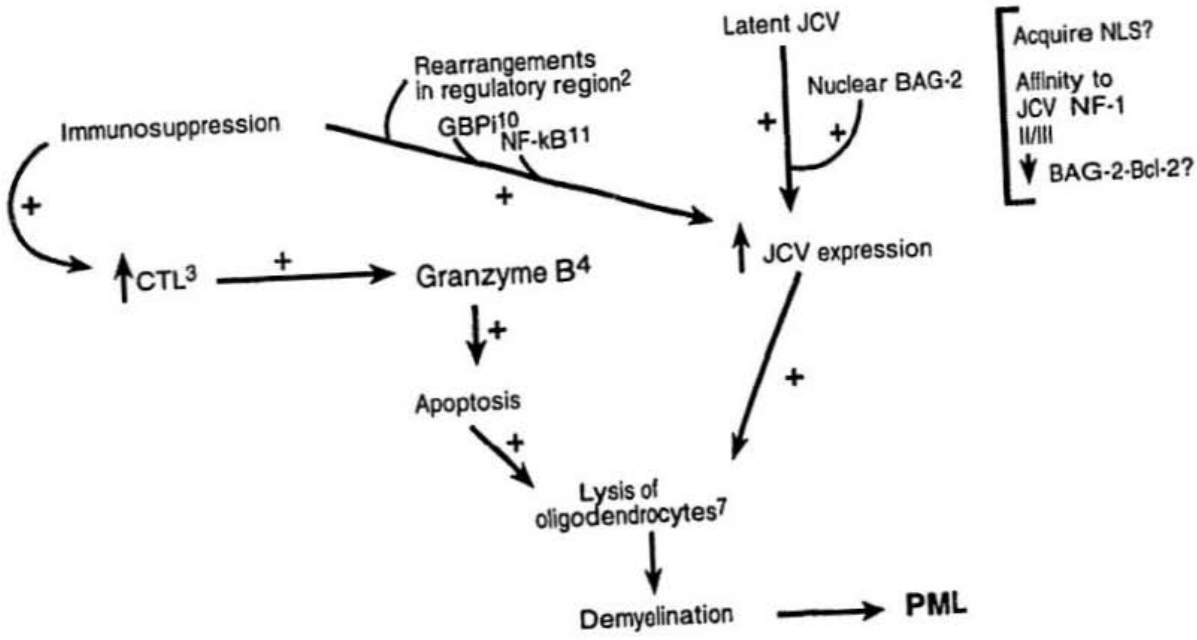
Kidney and B cells

A.



Brain cells

B.



into the nucleus may render these cells susceptible to apoptosis. The lytic infection of oligodendrocytes was shown to produce demyelination, the characteristic feature of PML (Trapp et al., 1988; Major et al., 1992). Though my results mirror this model, many aspects remain to be tested before an overall conclusion can be drawn. The model of Fig. 4.1 shows how BAG-2 may affect JCV latency and pathogenicity. In kidney and B cells, JCV remains latent. This can be explained by the presence of cytoplasmic BAG-2, which inhibits the apoptosis by negatively regulating p53 (Fig. 3.21). The inducer of apoptosis is p53, which is shown to be present at higher levels in JCV-infected cells (Ariza et al., 1994) as a result of DNA damage arising from JCV infection (Dorries and Elsner, 1991; Flaegstad et al., 1991). p53 may induce apoptosis by increasing the levels of bax (Miyashita and Reed, 1995) or by decreasing the levels of bcl-2 (Miyashita et al., 1994a), culminating in apoptosis. This apoptosis is counteracted by both BAG-1 (Takayama et al., 1995) and BAG-2. As a result the virus-infected cell survives for a prolonged time (for reviews, see Hockenbery, 1995; Korsmeyer, 1995). Thus, JCV remains latent in JCV-infected kidney and B cells (Fig. 4.1 A).

The effects of BAG-2 are distinct in brain cells. Reactivation of latent infection is seen under immunocompromising conditions, which then leads to haematogenous spread of JCV to brain (for review, see Major et

al., 1992). Thus, in the event of immunosuppression, JCV gets expressed in the brain. Expression may be due to: a) the brain-specific rearrangements in the JCV regulatory region, which allow efficient expression in brain cells (Ault and Stoner, 1994); b) cytokine-inducible GBPi and NF-κB transcription factors were shown to enhance JCV expression (Ranganathan and Khalili, 1993; Raj and Khalili, 1994) and c) the nuclear BAG-2 in brain cells, which would also activate JCV expression (this study). The activation of expression of JCV may lead to productive infection and lysis of the oligodendrocytes (for review, see Major et al., 1992). The other possibility is that the cytotoxic T lymphocytes (CTLs), which were seen in increased numbers in immunosuppressive conditions, like AIDS (for reviews, see Berke, 1994 and 1995) attack the virus-infected cells and cause apoptosis. The CTLs secrete a protease, Granzyme B/fragmentin, which induces DNA fragmentation and leads to apoptosis (Heusel et al., 1994). Lysis occurs as a consequence via this pathway. Loss of the oligodendroglial cells, which are required for myelination (for review, see Brophy et al., 1993), results in demyelination and the consequent pathology called PML (Fig. 4.1 B).

Another objective of this study was to examine the effect of BAG-2 on the regulation of the p53 promoter. As discussed in the Introduction, Bcl-2 was shown to inhibit the apoptosis induced by p53. How Bcl-2 accomplishes this was

unknown, since Bcl-2 does not have any functional motifs for biochemical functions that inhibit p53-induced apoptosis. As p53 is known to induce apoptosis by increasing bax mRNA levels and decreasing that of bcl-2 (Miyashita and Reed, 1995; Young and Korsmeyer, 1993), Bcl-2 protein, Bcl-2-related factors or Bcl-2 interacting factors may have a role in regulating p53. I hypothesized that BAG-2 is a Bcl-2-interacting protein (Fig. 3.20) and acts as a transcription factor (Fig. 3.12). These functions may be important for regulating the level of p53 transcription. In vivo assays showed that BAG-2 down-regulated the expression of p53 promoter by 4-fold (Fig. 3.21).

Generally, the present study did not completely clarify how BAG-2 negatively regulated the p53 promoter. One possibility is the binding of BAG-2 to the NF-1 site of p53 located at the transcription initiation site (Ginsberg et al., 1990) may sterically interfere or functionally repress the initiation of transcription. These results also rule out the possibility of a non-specific effect of BAG-2 on p53 promoter since pRc/CMV vector alone had no effect on p53 promoter.

My studies discovered the squelching of the expression of JCV (Fig. 3.15). Many eukaryotic and viral transcriptional activators were shown to activate transcription via novel factors called adaptors (Berger et al., 1990 and 1992; Kelleher et al., 1990; Martin et al., 1990; Martinez et al., 1991). A second possibility is that BAG-2 may act via a higher order, indirect interaction with the transcriptional

machinery. Specifically, adaptors act as a bridge between the upstream activator system and the basal transcription machinery. Overexpression of activators may titrate adaptors to function as repressors of transcription. Recently, an adaptor protein factor was purified and shown to interact with the VP16 activation domain and CTD tail of RNAP II (Kim et al., 1994). My studies showed squelching in P19 glial cells but not in U87 MG cells. This also supports the possible presence of cell-specific adaptors. If the putative adaptor is important for squelching of JCV expression by BAG-2, then this may explain why BAG-2 didn't exhibit squelching in U87 MG cells. The adaptor may be absent in U87 MG cells or non-functional, however, U87 MG cells also had no BAG-2 mRNA (Fig. 3.5). Though BAG-2 is ubiquitous, its location and association with other proteins may modulate its transactivation properties. Since ubiquitin-like domains were shown to mediate protein-protein interactions (Toniolo et al., 1988), it is possible that BAG-2 may interact with such putative adaptors via the same domain. Similarly, p53 was shown to repress many cellular and viral promoters (for review see Mercer, 1992), not by binding to promoter sequences, but by sequestering/squelching the factors essential for transcription (Pietenpol and Vogelstein, 1993).

CHAPTER 5

FUTURE DIRECTIONS

The regulation of gene expression is known to occur at the transcriptional, posttranscriptional and translational levels. The regulation of the first level, transcription, constitutes the most important of the three. Further, transcription factors that are specific to each tissue for gene expression are known to govern the expression of only a particular subset of genes. Thus, only a select cell-specific class of genes are transcriptionally activated, whereas others are transcriptionally repressed. Gene expression in brain tissues also is regulated mainly at the level of transcription. However, understanding brain-specific gene expression is difficult, because the brain is complex in function and in tissue diversity. Much attention recently has been focused on the human neurotropic virus, JCV, because JCV is strictly brain-specific in its expression and serves as a genetically well defined system.

The JCV regulatory region is contained mainly in two 98 bp tandem repeats, that are bound and regulated by several *trans*-acting factors (Fig. 1.2). Studies from our and other laboratories indicated the importance of NF-1 motifs in the glial cell-specific expression of JCV (Kumar *et al.*, 1993). Subsequently, a cDNA encoding the factor that binds to the NF-

1 sequences was isolated and shown to activate the expression of JCV in non-glial cells (Kumar, 1994). However, no structure-function studies were done. My studies presented here demonstrated the importance of the factor encoded by this cDNA, BAG-2, and its interaction with the JCV NF-1 II/III motifs, in JCV expression.

BAG-1 was ubiquitously expressed, but the C-terminus specific to BAG-2 was restricted to P19 cells (Fig. 3.5). Interestingly, the 5' leader sequences appeared to be cell-specific, as revealed by PCR analysis of RNA from different cell types (data not shown). Further, BAG-2 belongs to a family of proteins arising by alternative splicing of a single primary transcript, as revealed by Southern blot (data not shown). One attractive continuation of my studies, would be to clone the 5' sequences by 5' RACE-PCR as described (Apte and Siebert, 1993). Studying the role of specific 5' sequences may illuminate the mechanism for the translational control of BAG-2 gene expression in different cells. Regulating the expression of the effector proteins at the translational level may be important for an effective mechanism that allows the rapid response that is important in various brain functions response.

My studies showed that BAG-2 was localized in the nucleus in P19 glial cells only. A protein(s) that interacts with the NLSs of BAG-2 may be restricted to P19 glial cells. In the future, such proteins might be identified by cross-linking and

ligand-binding assays, as described in other studies (Adam et al., 1989; Li and Thomas, 1989). Alternatively, anti-idiotype antibodies specific to the NLS of BAG-2 could be generated to identify the proteins that specifically interact with NLS of BAG-2, as described for the NLS of SV40 T-antigen (Stochaj et al., 1990). Relevantly, the SV40 T-antigen NLS was shown to interact with different proteins from different cell types (Stochaj et al., 1990). To date, my studies did not clarify whether or not NLS is important for nuclear localization. Therefore, the NLS function can next be addressed by domain swapping with various proteins, by deleting, by mutating the NLS. After this would follow testing for the subcellular localization of the various recombinant proteins and the mutated BAG-2 proteins in the BAG-2-deficient cell line, U87 MG.

Though my studies narrowed the putative sequences for the DNA-binding and transactivation domains to the central coiled-coil and C-terminus, the residues which are important for these activities were not identified or functionally characterized. Therefore, site-directed mutagenesis can address these issues in the future using the methods previously descrived (Kunkel et al., 1987). Further, the swapping of domains with various proteins, could be used.

Earlier studies from our laboratory, showed that JCV T-antigen transactivated JCV_L and the hypothesis that this occurred when the T-antigen facilitated the binding of NF-1

was formulated (Kumar, 1994). Since BAG-2 also transactivated JCV_L in my studies, it is essential to observe the putative interaction between BAG-2 and T-antigen to test this hypothesis. Therefore, the next step could be to assay for this interaction, using the yeast two-hybrid system, as described (Fields and Song, 1989). Most DNA viruses carry their own "anti-death" gene to protect the infected cells from PCD. JCV appeared to be unique-instead of having an anti-death gene, JCV may instead exploit the counter-apoptotic properties of BAG-2. Though BAG-2 regulates JCV expression in the brain, a mechanism for BAG-2 allowing JCV to remain latent in other organs, such as in kidney and B cells is unknown. This mechanism could be addressed in future studies, by isolating the proteins that interact with BAG-2. The methods of choice are the yeast two-hybrid system or protein-affinity chromatography. For the latter, complexes with BAG-2 would be isolated and identified by passing the WCEs from kidney and B cells through a column containing the immobilized GST-BAG-2 fusion protein, as described (Formosa et al., 1991).

My studies shown in Fig. 3.15 indicated that squelching occurred when BAG-2 was overexpressed in P19 glial cells. Therefore, the involvement of a transcriptional mediator/adaptor/coactivator was indicated. A future direction is to test, isolate and characterize the putative coactivator between BAG-2 and the basal transcription machinery. Again, the putative factor can be tested and purified by protein-

affinity chromatography with GST-BAG-2 fusion protein, as described above. Such studies are important to evaluate the possible scenario that such an adaptor is the limiting factor in the kidney and B cells. Then, the latency of JCV in these cells could be explained. The specificity of such a factor in glial cells of the CNS could restrict the JCV expression and pathology of PML.

The results in Fig. 3.21 showed that BAG-2 repressed the p53 promoter. This repression may be achieved by binding to the p53 NF-1 motif. In future studies, to clarify how BAG-2 might have repressed the p53 promoter, the following avenues could be taken. In vitro transcription assays with increasing concentrations of my recombinant BAG-2 protein in the presence and absence of JCV NF-1 oligonucleotide could be undertaken using the methods described previously (Kelleher et al., 1990). The results would address whether BAG-2 repressed the p53 promoter by indirectly sequestering the adaptors or coactivators from the transcriptional machinery. Alternatively, the mechanism could be direct interaction with the p53 NF-1 motif resulting in repression. This can be tested by mutating the p53 NF-1 motif, as described previously (Kunkel et al., 1987).

My studies presented here may address the brain-specific expression and resulting pathology in the CNS and latency in other target tissues. However, many hypotheses require testing before a final conclusion can be presented. If the above

mentioned hypotheses were proven to be correct, then BAG-2 has a potential for PML therapy.

In addition to the implications for the pathology of JCV, my studies have identified a novel transcription factor, BAG-2. This cellular protein clearly introduces many exciting possibilities. The confinement of the brain requires strict control of growth by mechanisms such as apoptosis. Therefore, certain non JCV-related studies in the future may take the following directions; 1) Examination of the distribution of gene expression in various organs using commercially available systems. Such systems could be exploited to uncover the importance of BAG-2 in specific organs. Immunofluorescence studies of the interesting cell types would be a priority in subsequent studies of cell specificity. 2) "Zoo" blots can be similarly examined to evaluate the role of BAG-2 in different species. The organs of interesting and well-studied species could be examined. Such studies could address the role of BAG-2 and homologues in apoptosis for various species across the phylogenetic spectrum. 3) The brain-specificity, and possible specificity to other tissues, of BAG-2 could be used to study many general features of the cellular and molecular biology aspects of the exciting process of apoptosis (for review, see Hockenbery, 1995).

CHAPTER 6

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