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The transcriptional enhancer element, κB, regulates promoter activity of the human neurotropic virus, JCV, in cells derived from the CNS

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ABSTRACT

Studies on the regulation of the human neurotropic virus (JCV) promoter, have been focused primarily on the 98 bp tandem repeat sequence which confers glial-specificity to viral gene expression. We demonstrate that a distinct regulatory element outside of the 98 bp region, which spans a stretch of 10 nucleotides (nt) (5′-GGGAATTTCC-3′) increases transcriptional activity of JCV late (JCV_L) and early (JCV_E) promoters in glial cells. Sequence analysis of this motif reveals extensive homology to the κB sequence of HIV-1 (5′-GGGACTT-TCC-3′). A DNA fragment corresponding to the 10 nt sequence of JCV exhibits transcriptional activity when placed upstream of the test promoter in glial cells. The induction mediated by this regulatory motif is moderately enhanced in response to phorbol 12-myristate 13-acetate (PMA) in glial cells. Band-shift and UV-crosslinking experiments suggest that glial cells constitutively produce proteins that specifically interact with the JCV κB, but not the HIV-1 κB motif. Treatment of cells with PMA results in formation of new complexes that are sensitive to the κB sequences derived from the JCV and HIV-1 genomes. These results suggest that the κB sequence located in the JCV genome may play a role in transcriptional regulation of JCV gene expression by interacting with inducible and uninducible nuclear proteins from glial cells.

INTRODUCTION

The JC virus (JCV), a member of the genus polyomavirus, is a human papovavirus etiologically linked to a fatal demyelinating disease of the central nervous system (CNS) termed Progressive Multifocal Leukoencephalopathy (PML) (1–12). This virus exhibits close sequence homology to the well-characterized human papovaviruses, simian virus (SV40), and the BK virus (BKV) (13, 14). JCV and BKV share approximately 80% homology between their viral early proteins (T- and t-antigens) and up to 80% homology between the late viral capsid proteins (VP1, VP2, and VP3). JCV and SV40 share approximately 70% homology in these regions. However, unlike BKV and SV40, JCV has a narrow tissue tropism that has hampered the study of this virus in culture. JCV replicates efficiently in primary human fetal glial cell cultures rich in spongioblasts, the presumed precursor of oligodendrocytes (15, 16). Several human cells (embryonic kidney, amnion, and urine-derived epithelium) have been shown to poorly support viral replication (17–19).

The greatest degree of divergence between JCV, BKV, and SV40 is in the viral control region which in JCV contains the origin of DNA replication, promoters for early and late gene transcription, and tandem 98-base pair (bp) direct repeats. Several lines of evidence indicate that the restricted tissue specificity of JCV is determined by this region of the virus. Analyses of viral gene expression by transient transfection studies and in transgenic animals have revealed that the tissue tropism of JCV resides, at least in part, in the glial-specific activation of the viral early and late promoters (20–23). Evidently, the 98-bp repeats of the JCV regulatory sequence function as tissue-specific enhancers for these promoters.

Our laboratory has initiated a detailed examination of the cis- and trans-regulatory factors that restrict JCV gene expression to glial cells. Studies by us (24–26) and others (27, 28) have suggested that the JCV control region contains multiple protein binding domains that interact with distinct factors present in glial and non-glial cells (24–28). Figure 1 illustrates the structural organization of the JCV regulatory region and the positions of DNA-binding proteins and cis-acting elements. Use of hybrid promoter constructs in transient transfection studies have revealed that at least two of these domains positively and negatively contribute to the specific transcription of a heterologous promoter in glial and non-glial cells, respectively. Thus, it is likely that transcription of the viral early promoter is under positive and negative regulation in permissive and non-permissive cells. In support of these findings, results from cell fusion experiments have indicated that expression of the viral early promoter was extinguished in the nuclei of glial cells when these cells were

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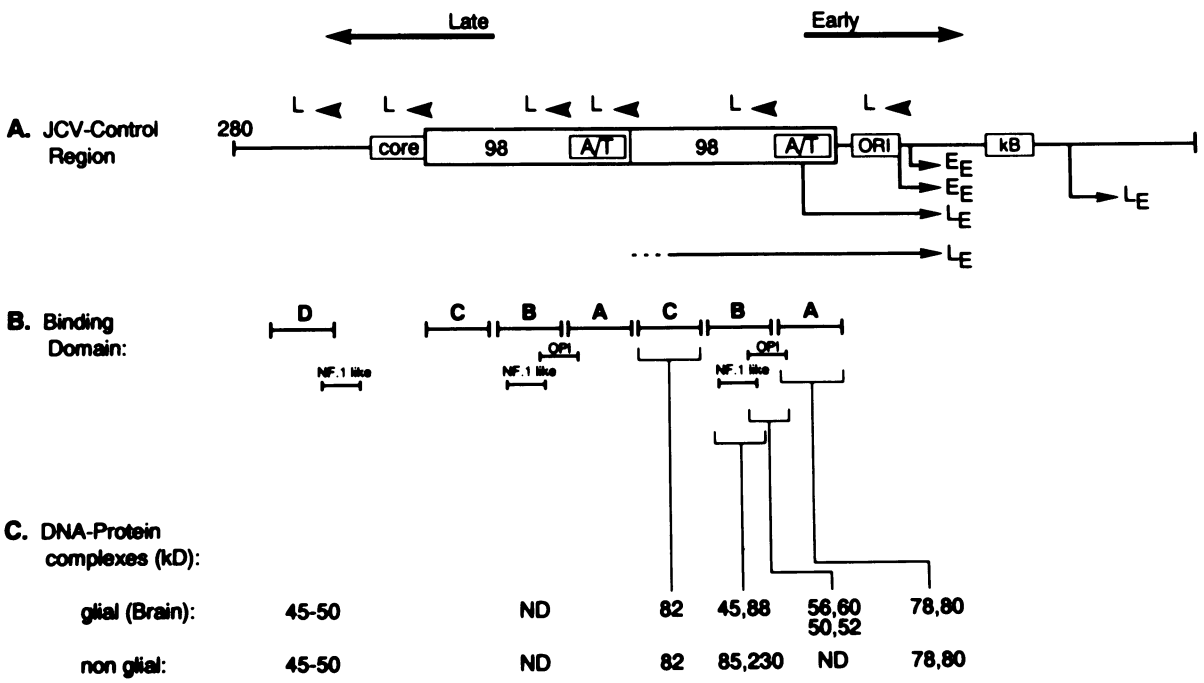

Figure 1. Schematic diagram of the transcription control region of JCV. **A.** Control elements including the origin of viral DNA replication (ori), the AT-rich (TATA box) sequence, the core region representing an octameric motif of SV40, (GTGCAAAG), and the κB box (GGGAATTTCC) are positioned between the early (encoding large T- and small T-antigens) and the late (encoding viral structural proteins $VP_{1, 2, \text{and } 3}$) translation initiation sites. The arrows indicate start sites of viral early RNAs before DNA replication (E_E) and after DNA replication (L_E) (41, 42). The positions of the late RNA initiation sites are shown by arrowheads (43). **B.** Locations of protein binding regions and cis-acting regulatory elements (24–28) as examined by various DNA-binding techniques and transient transfection assay. **C.** The relative molecular mass of the complexes formed in glial and non-glial nuclear extracts as examined by UV-crosslinking techniques (24). ND, not determined.

fused with fibroblasts (29). This observation suggests that a negative transdominant factor in non-glial cells directly or indirectly suppresses expression of the JCV early promoter.

Earlier studies by Major, et al (30) have identified a putative κB sequence on the early side of the viral origin (Figure 1). κB is a potent transcription enhancer which was first identified in the kappa-light chain of immunoglobulin gene, and later in many inducible cellular and viral transcription units (31). This enhancer, which is composed of 10 nucleotides (nt) (GGGACTTTCC), binds to a large family of proteins called Nuclear Factor-Kappa B (NFκB). NFκB is a complex of two proteins of 50 and 65 kD that is thought to bind DNA either as a heterodimer of P50/P65 or as a homodimer of P50/P50 (31). These proteins pre-exist in the cytoplasm of most cells in an inactive form complexed to an inhibitor, termed IκB (31). Stimulation by a number of agents such as phorbol-12 myristate-13 acetate (PMA), lipopolysaccharide (LPS), or tumor necrosis factor-α (TNFα), results in the dissociation of the IκB-NFκB complex, presumably by a protein kinase C-dependent phosphorylation of IκB (31). The unbound NFκB is, in turn, transported to the nucleus where it binds to its target sequence and exerts its activity.

In the present report, we have investigated the biological effects of the 10 nt κB sequence homologous to the κB transcription element of HIV-1 on the expression of the JCV early and late promoters in human glioblastoma cells. We now demonstrate that this κB sequence induces overall activities of the JCV late and to a lesser extent, early promoters in glial cells. Results from binding studies revealed the association of multiple inducible and non-inducible nuclear proteins from glial cells that differentially bind to JCV-κB and a classical κB sequence derived from the HIV-1 promoter.

EXPERIMENTAL PROCEDURES

Plasmid DNA constructs

The plasmids, $pJCV_L$ CAT and $pJCV_E$ CAT contain the 286 bp Hind III-Pvu II fragment representing the overlapping regulatory sequences of the JCV late and early genes, cloned upstream of the chloramphenicol acetyl transferase (CAT) gene (32). $pJCV_E$κB-CAT and $pJCV_L$κB_CAT plasmids were constructed by placing a 388 bp polymerase chain reaction (PCR) product representing the JCV regulatory region upstream of the CAT gene in the pBL-CAT_3 vector (33). The primers used in the PCR were 5′-CCTCCCTATTCAGCACTTT-3′, and 5′-GGCTCG-CAAAACATGTTCCC-3′. The template used to amplify DNA differs from the published sequence for MAD-1 strain of JCV in that it contains a small deletion in the second 98 bp repeat (nt 110–132). Such disparate nt alterations are apparently a common feature of JCV (K. Dorries, personal communication) and do not affect cell-specific transcription of viral promoters in glial cells.

Deoxyoligonucleotides of JCV-κB (5-GGGAATTTCC-3′) were synthesized using phosphoramidite chemistry and purified by gel electrophoresis. Complementary oligonucleotides were treated with polynucleotide kinase, annealed, and cloned into the pBL-CAT_2 expression vector at the Sal 1 site upstream of the HSV-TK promoter (33). The presence of the oligonucleotide insert was verified by DNA sequencing (34).

Transient transfection and CAT assay

Human glioblastoma cells, U-87MG (ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco). Cells were seeded at

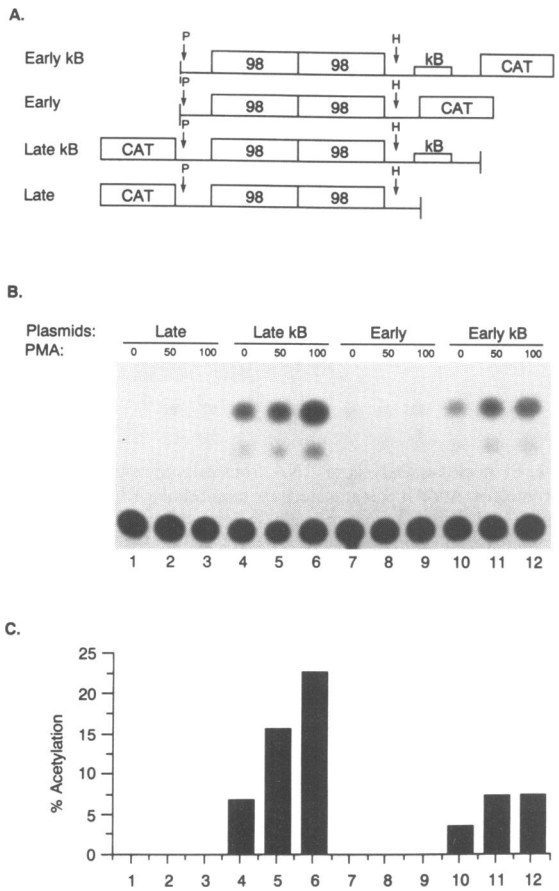

Figure 2. Transcriptional activity of JCV-κB in glial cells. **A.** JCV_E and JCV_L constructs contain a 288 bp Hind III-Pvu II DNA fragment of the JCV regulatory sequence in early and late orientations, respectively, placed in front of the CAT gene (32). JCV_E κB and JCV_L κB constructs contain a 388 bp DNA sequence from JCV that includes a 286 bp stretch from the Hind III site to the Pvu II site plus 102 nt overlapping the early side of the replication origin. The 388 bp DNA fragment was generated by polymerase chain reaction (PCR) with appropriate primers in which Bam H1 restriction sites were incorporated as cloning sites. **B.** Approximately 5×10^5 human glioblastoma cells (U-87MG) were transfected with 4 μg of plasmid DNA by Ca-phosphate precipitation technique (35). After 4 hours, cells were washed with PBS and refed with fresh medium containing 50 or 100 ng/ml PMA. Cell extracts were prepared 36–48 h post transfection and CAT enzymatic activity was determined (37). **C.** The percent conversion of chloramphenicol to its acetylated forms was determined by liquid scintillation counting of substrate and acetylated forms.

5×10^5 cells per 60 mm plate and 20 h later 4 μg of test plasmid DNA was introduced into the cells by the Ca-phosphate precipitation procedure (35). The final amount of DNA in each transfection mixture was brought to 15 μg with pUC DNA.

At 4 h post-transfection, cells were subjected to glycerol shock (36). Cells were washed several times with PBS and incubated with fresh medium in the absence or presence of PMA at concentrations of 50 or 100 ng/ml. All experiments were performed independently at least three times. Cells were harvested at 36–48 h post-transfection and assayed for CAT activity as described (37). Percent conversion of [^{14}C]chloramphenicol to its acetylated form was determined by liquid scintillation counting of the spots cut from the TLC plates.

Nuclear extract preparation and band-shift assay

Nuclear extracts were prepared from U-87MG cells as described previously (38). For band-shift experiments, the oligonucleotide probe containing the JCV-κB sequence (5′-GGGAATTTCC-3′) was end-labeled with [γ^{32}P]ATP, annealed, and purified on a 9% native acrylamide gel. Approximately 15,000 cpm of probe was incubated with approximately 10 μg of nuclear extract protein in the presence of 25 mM Hepes (pH 7.9), 40 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 0.4 mg/ml BSA, 3 mM GTP, 0.3 mg/ml poly[dI-dC], and 10% glycerol (v/v) in a final volume of 30 μl at 4°C for 30 min. Competition experiments were carried out in the presence of unlabeled JCV-κB, HIV-1-κB, and HIV-1-κB^{mut} DNA fragments. In this case, the extract was preincubated with the competitor DNA for 10 min prior to the addition of the labeled probe. Complexes were electrophoresed on 6% polyacrylamide gel (prerun at 150 v for 2 h) at 150 v for 4–5 hours with continuous circulation of running buffer at 4°C. Gels were fixed in 10% acetic acid, 10% methanol, dried and exposed to Kodak X-AR film at −70°C with intensifying screen.

UV-crosslinking

Labeled oligonucleotides (JCV-κB) were mixed with extracts under conditions as described in the band-shift assay. Reaction mixtures were exposed to UV light (302 nm) for 30 min at 4°C and resolved by SDS-PAGE. The gel was dried and autoradiographed as described to determine the relative molecular mass of the complexes.

RESULTS AND DISCUSSION

Activation of JCV promoters by κB sequence in glial cells

To gain insight into the glial-specific expression of the JCV genome, and to examine the possible role of the sequence located downstream from the enhancer/promoter, we carried out a series of transient transfection experiments utililizing plasmids containing the reporter chloramphenicol acetyl transferase (CAT) gene fused to various regions of the JCV regulatory sequence. Figure 2A schematizes the structure of the recombinants which were used in these studies. The constructs were introduced into human glioblastoma cells (U-87MG) by the Ca-phosphate precipitation method (35) and CAT enzymatic activities were measured 48 h after transfection. The results shown in Figure 2B indicate that the sequence positioned downstream from the 98-bp repeat increases transcription of viral late and early promoters by 10- and 5-fold, respectively, in glial cells (compare lanes 1 and 7 with 4 and 10, respectively). Inspection of the sequences located upstream of the 98-bp repeats has revealed a stretch of 10 nt (5′-GGGAATTTCC-3′) with significant homology to the κB enhancer of HIV-1 (5′-GGGACTTTCC-3′). It is now well-established that the κB sequence binds to a family of transcription factors which are induced with a variety of agents, such as phorbol esters, and enhances transcriptional activity of the linked promoter (31). To examine the inducibility of the JCV-κB sequence, cells were maintained in medium containing phorbol-12-myristate B-acetate (PMA) after transfection, and transcriptional activities of the JCV early and late promoters were examined by CAT assay. As shown in Figure 2B, treatment of cells with PMA revealed approximately 2- to 3-fold elevation in the activity of the κB-containing constructs (lane 5, 6, and 11, 12). In parallel studies, the κB sequences show less than two-fold enhancement of the JCV early and late promoter activity in Hela cells (data not shown). Treatment of these cells with PMA had no effect on viral early and late promoter activities. These studies suggest that the upstream sequence of JCV encompassing

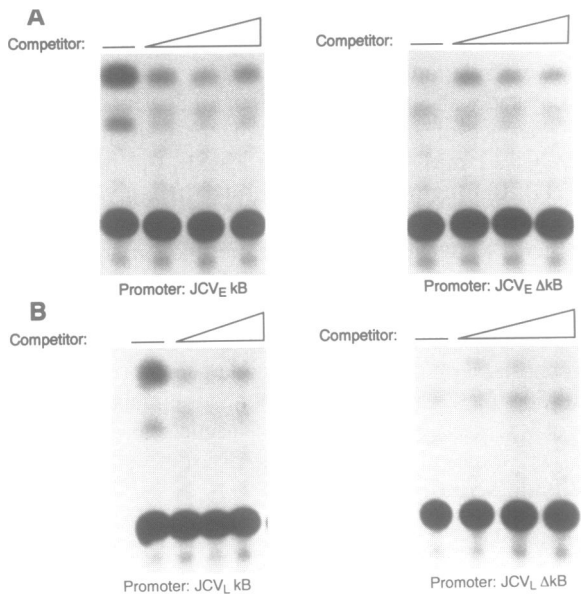

Figure 3. Co-transfection competition analysis of the JCV promoter in U-87MG glial cells. The JCV late and JCV $\varkappa$B reporter constructs containing various lengths of the JCV early and late regulatory sequences, respectively (as detailed in Figure 2A) were transfected alone or together with increasing amounts (250-, 500-, and 1,000-fold molar excess) of competitor oligonucleotide fragment containing the JCV $\varkappa$B sequence (5′-GGGAATTTCC-3′). Cellls were harvested 48 h post-transfection and CAT assay was determined (37).

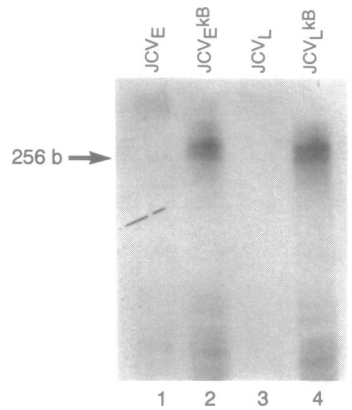

Figure 4. S1 nuclease analysis of RNA from cells transfected with JCV$_E$ and JCV$_L$ promoters. At 48 h post transfection, total cellular RNA was isolated from cells transfected with constructs containing JCV$_E$ and JCV$_L$ and hybridized to a single-stranded uniformly labeled CAT DNA probe. S1 digestion was carried out by the method described previously and protected DNA fragments were purified and analyzed by electrophoresis in a denaturing sequencing gel as described previously (39). The arrow indicates the position of the 256 nt protected fragment.

the $\varkappa$B motif has the capacity to augment the basal activity of viral early and late promoters in U-87MG glial cells. Evidently, this sequence responds positively to NF$\varkappa$B inducers such as PMA. However, the modest induction of these promoters upon PMA treatment suggests the involvement of a trans-regulatory factor(s) that is constitutively active in these cells.

Next, to determine more specifically whether the $\varkappa$B sequence plays a role in the activity of the intact JCV promoters, in vivo competition experiments were carried out. To this end, the synthetic oligonucleotide derived form JCV $\varkappa$B was co-transfected with the reporter constructs containing JCV early and late regulatory sequence and the level of CAT activity was determined. In the presence of 250-, 500-, and 1,000-fold molar excess of $\varkappa$B sequence, JCV early and late promoter activities were substantially reduced (Figure 3) suggesting that the $\varkappa$B sequence is important for enhanced transcriptional activity of JCV promoters and that there are other regions of JCV that are necessary to maintain viral gene transcription at high levels in glial cells under similar conditions. The $\varkappa$B sequence exhibits no significant effect on transcriptional activity of viral promoters with no $\varkappa$B sequence (Figure 3). These data imply that the $\varkappa$B sequence is operative in the context of the viral promoter, and increases both early and late promoters.

Transcription of the JCV promoters in glial cells

To verify that the increase in CAT activity of the $\varkappa$B containing plasmids was due to increased levels of CAT transcripts, the level of CAT RNAs was measured by S1 nuclease protection assay (39). In this study, a 458 nt single-stranded S1 probe containing a sequence complementary to the CAT coding sequence was uniformly labeled and hybridized to total RNAs derived from cells transfected with various JCV constructs (as shown in Figure 4). S1 nuclease digestion by CAT RNA is expected to result in a protected fragment of 256 nt. Results shown in Figure 4 illustrate the protection of an appropriately cleaved 256 nt DNA fragment corresponding to protection by RNA transcribed only in cells transfected with the $\varkappa$B plasmids. These results demonstrate that the $\varkappa$B-sequence stimulates transcription of JCV early and late promoters in glial cells.

Activation of a heterologous promoter in glial cells by JCV-$\varkappa$B sequence

To further examine the transcriptional activity of the JCV-$\varkappa$B sequence, we synthesized a double-stranded oligonucleotide harboring the JCV-$\varkappa$B sequence, GGGAATTTCC, and fused it to the 5′-end of a test promoter derived from Herpes simplex virus thymidine kinase (TK) (33) in both early and late orientations, separately. This promoter contains several transcriptional elements, i.e. TATA box, GC-rich sequences and a CAAT box (40). Transfection experiments were carried out in the presence or absence of PMA, and CAT enzyme production was measured after 48 h. As shown in Figure 5, basal CAT activity, due to expression of the TK promoter, was significantly increased in glial cells when the JCV-$\varkappa$B sequence was linked to the test TK promoter. The level of enhancement was significantly higher when the $\varkappa$B sequence was placed in the late orientation. Treatment of the cells with PMA showed only a two-fold increase in the CAT activity of constructs containing $\varkappa$B in the late orientation and had an insignificant effect on the construct with $\varkappa$B in the early orientation. A low level of induction ($<50\%$) of TK promoter activity was detected in Hela cells transfected with the hybrid promoter harboring JCV-$\varkappa$B in the late orientation (data not shown). These results suggest that the $\varkappa$B sequence of JCV is operative and has the capacity to increase transcriptional activity of a fused heterologous promoter in glial cells.

Formation of specific DNA protein complexes by the JCV-$\varkappa$B sequence

In an attempt to identify the cellular factors present in glial cells that interact with the JCV-$\varkappa$B sequence, band-shift experiments

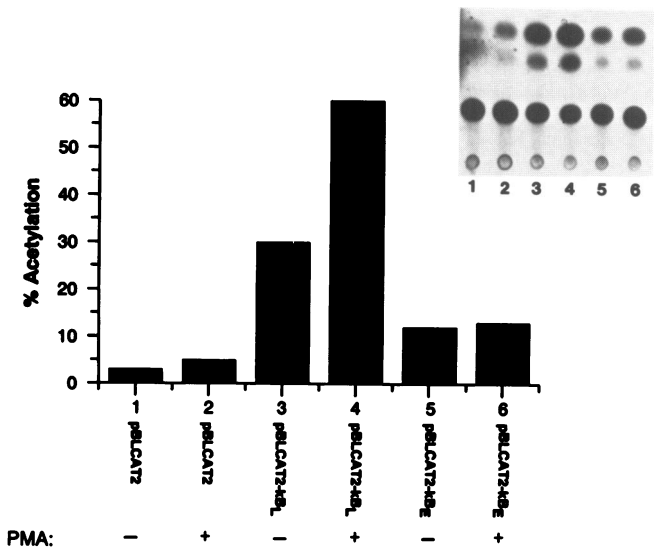

Figure 5. Functional analysis of the JCV-κB sequence. Synthetic double-stranded DNA (oligo-JC κB) homologous to the JCV-κB sequence was placed in either orientation, in front of the Herpes simplex virus thymidine kinase gene promoter which contains a TATA box, GC-rich sequence and CAAT box (40). The control and each of the recombinant constructs were introduced into U-87MG glial cells in the presence or absence of PMA by the Ca-phosphate precipitation procedure (35) and enzymatic activity of the CAT protein expressed from these plasmids was determined (37). The percent of chloramphenicol converted to its acetylated forms was determined as described in Figure 2 legend.

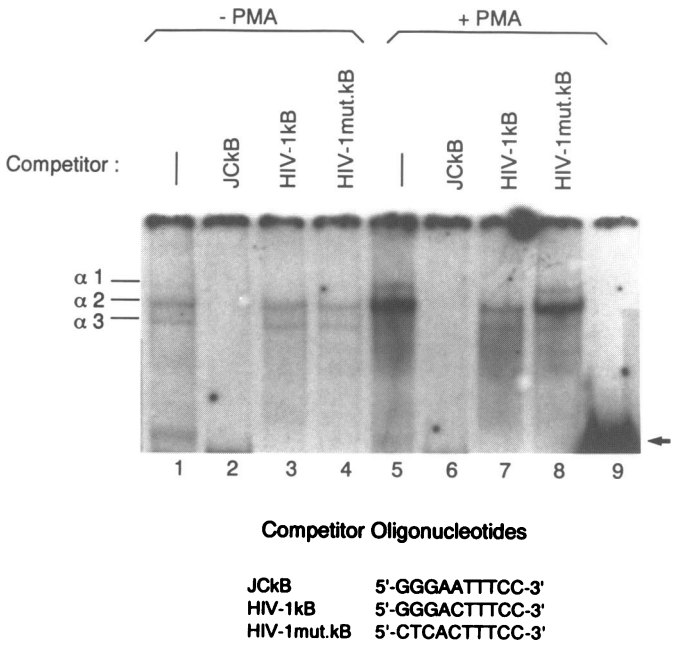

Figure 6. Protein-binding analysis of the JCV-κB sequence. Nuclear extracts were prepared from U-87MG glial cells that were treated with PMA as previously described (31). Approximately 15,000 cpm of [^{32}P]-end-labeled double-stranded oligonucleotide fragment were incubated in 30 μl of nuclear extract reaction mixture containing 10–12 μg of protein as described earlier (36). The resulting complexes were resolved on a 6% native polyacrylamide gel. In competition studies, unlabeled DNA fragments (>100-fold molar excess) were added to each reaction prior to incubation with the probe. Lane 9 represents probe alone.

were performed. In this study, double-stranded JCV-κB oligonucleotide was end-labeled by T4-polynucleotide kinase in the presence of [γ^{32}P]ATP and incubated with nuclear extracts derived from U-87MG cells in the presence or absence of PMA. The extract from untreated cells produced two closely migrating complexes, α_2 and α_3. Addition of excess unlabeled JCV-κB oligonucleotides (>100-fold) abolished formation of these complexes, whereas under similar conditions, the κB sequence from the HIV-1 promoter containing only one nucleotide mismatch revealed no significant effect on the intensity of the α_2 and α_3 bands (Figure 6, lanes 1–3). Expectedly, the mutant HIV-1-κB oligonucleotide (GGGACTTTCC changed to CTCACTTTCC), which is functionally inactive (31), was unable to block the formation of the α_3 complex (Figure 6, lane 4). The PMA-treated glial extract showed an elevated level of α_2 complex (or a complex that co-migrates with α_2) and the appearance of a new complex α_1 (Figure 6, lane 5). All three forms (α_1, α_2, and α_3) were sensitive to excess amounts of unlabeled homologous competitor sequence. Preincubation of the extract with HIV-1-κB competitor reduced the intensity of the α_2 complex to the level seen in untreated extract, and abolished formation of the α_1 complex. The addition of unlabeled HIV-1-κB mutant to the extract showed no significant effect on the intensity of the α_1, α_2, and α_3 complexes (Figure 6, lane 8). These results suggest that, in addition to the two inducible complexes which recognize both JCV and HIV-1-κB sequences, glial cells may contain nuclear proteins non-responsive to PMA that preferentially recognize the JCV-κB sequence.

Identification of JCV-κB binding proteins by UV-crosslinking

To further characterize the nuclear proteins that participate in binding to the JCV-κB sequence, UV-crosslinking experiments were employed. Binding reactions were carried out according to the band-shift procedure as described above using 20 μg of nuclear protein from U-87MG and PMA-treated U-87MG cells. Complexes were cross-linked for 30 min at 4°C using long wave UV light (302 nm). The resulting complexes were directly analyzed by SDS-PAGE. As shown in Figure 7, three major complexes, a, b, and c, migrating as broad bands in the ranges of 35, >68, and 56 kD, respectively, were reproducibly detected

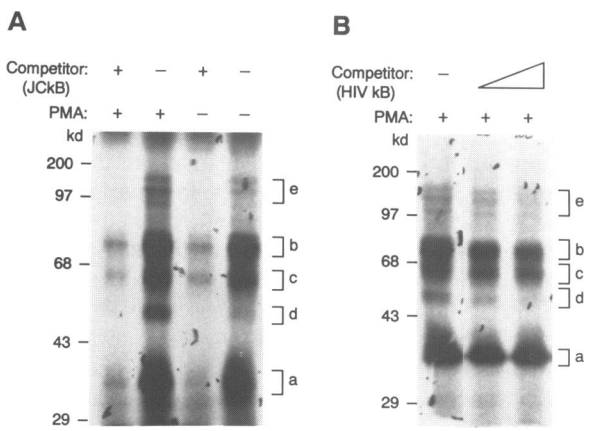

Figure 7. Identification of the κB binding protein by UV-crosslinking. UV-crosslinking and competition assays were carried out as described previously (36). Briefly, the double-stranded JCV κB oligonucleotide was uniformly labeled and UV-crosslinked to nuclear protein derived from glial cells PMA-treated and untreated in the presence or absence of unlabeled JCV κB (A) and HIV-1 κB (B) sequences.

in the untreated extract. In the presence of PMA, additional complexes, d and e, with relative molecular masses of 48 and 87–102 kD, respectively, were observed. Addition of 100-fold excess of unlabeled JCV-κB DNA significantly reduced the intensity of the bands corresponding to all five complexes (Figure 7A, lanes 2 and 4). Preincubation of the extracts with unlabeled HIV-1-κB DNA reduced the intensity of the inducible 48 and 87–102 kD complexes, but showed virtually no effect on the formation of 35, >68, and 56 kD complexes (Figure 7B).

At the present time, the role of these complexes in the activation of the JCV promoter in glial cells is unclear. However, the profile of transcription activation of the viral promoters containing the JCV-κB sequence upon PMA treatment, and the formation of specific NFκB complexes in PMA-treated extract, suggest that the participant proteins in the 35, >68, and 56 kD complexes may be involved in the overall activation of the JCV early and late promoters. Similarly, the proteins that form the 48 and 87–102 kD complexes may mediate transcription enhancement of JCV gene expression upon PMA induction.

In summary, experiments described here demonstrate that the sequences homologous to the NFκB transcriptional element present within the JCV genome have the capacity to stimulate expression of the bidirectional viral promoter in glial cells. This regulatory element, which differs in only one nt with the classic κB sequence of HIV-1, binds to constitutive and inducible DNA binding proteins present in glial cells. Experiments are in progress to examine the importance of this regulatory motif in the physiology of the JCV life cycle in cells treated with a variety of agents, such as LPS, cAMP, cytokines, tumor necrosis factors α and β (TNFα and TNFβ), transforming growth factor β (TGFβ), and interleukin 1. Results from these studies should enhance our current knowledge of the mechanisms that trigger replication and/or expression of latent JCV in immunocompromised individuals.

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