Regulation of a human neurotropic virus promoter, JCV_E: identification of a novel activator domain located upstream from the 98 bp enhancer promoter region

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ABSTRACT

Transcription of the human neurotropic virus promoter, JCV_E, and its regulation in glial cells are controlled by the 98 bp tandem repeats positioned between the viral early and late genes. Here, we show that a region, designated domain-D, located upstream from the 98 bp repeats functions as a transcriptional activator and increases JCV_E promoter activity. Using the reporter $SV40_E$ promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene, we demonstrate that domain-D stimulates the basal $SV40_E$ promoter activity in glial and to a lesser degree in HeLa cells. Results from gel mobility-shift assays indicate that domain-D interacts with proteins derived from glial and HeLa extracts and results in the formation of specific DNA-protein complexes. Through UV cross-linking assays, we demonstrate that these complexes have similar electrophoretic mobilities which comigrate with the 43 – 50 Kd proteins derived from glial and HeLa cells. These findings, together with our previous observations, imply that the JCV_E control region is composed of multiple common and specific activator domains that may account for the increased expression of the promoter in glial cells. The possible role of the D-binding protein in transcription of the JCV_E promoter is discussed.

INTRODUCTION

Temporal and tissue specific expression of genes in eukaryotic cells is largely regulated at the level of transcription (1). The control region of the polymerase II transcription unit contains modular arrays of cis-acting elements located near the start of transcription: the TATA box, a variety of upstream regulatory sequences located within 50 to 400 base pairs of the start site, and enhancer elements that may be located at great distances upstream or downstream of the start site (2 – 10). Transcriptional activation occurs, in part, through binding of cellular transcriptional factors to these cis-acting elements contained within the promoter and enhancer, and through protein-protein interactions between activators (7). Thus, genes can be selectively expressed according to the relative abundance of these specific transcriptional factors found in cells and the particular arrangement of factor-binding sites present in their regulatory regions.

The regulation of the human neurotropic virus promoter, JCV_E, expression in glial cells represents an attractive model system for studying cell type-specific mechanisms of transcriptional control. JC is a human papovavirus which selectively replicates in glial cells (11). This virus has been repeatedly isolated from demyelinated plaques in the brains of patients with progressive multifocal leukoencephalopathy (PML) and is suspected to be the causative agent of this disease (12 – 18). The structure of the control region of JCV contains two 98 base pair tandem repeats, shown by transient expression assays to be responsible for its unique tissue tropism (19, 20, 21). Using band-shift and UV-cross linking techniques, multiple protein-binding domains, each interacting with distinct nuclear proteins from HeLa and human fetal brain extracts, have been identified (22). These binding domains positively contribute to the specific expression of a heterologous promoter in glial cells (23). One of the most active domains, designated 'B', is located in the center of the 98 bp repeat and interacts with a major 45Kd and a minor 85 Kd protein derived from brain cells. The purified 45Kd protein was shown to stimulate *in vitro* transcription of the JCV_E promoter in extracts prepared from non-glial HeLa cells (24). Using deletion mutation analysis, a silencer region (designated 'OP') within each 98-bp repeat, juxtaposed to the B-domain, has recently been identified in our laboratory. These observations suggest that a modular arrangement with combinations of several sequence elements within the 98bp enhancer/promoter repeat may be responsible for the unique program of glial-specific transcription of JCV_E promoter.

In this study, we report the identification of a novel cis-acting regulatory sequence that positively affects transcription of the JCV_E promoter in extracts derived from glial and non-glial cells. This domain, designated 'D', is located upstream of the 98 bp enhancer elements in the distal position of the early RNA initiation site and has the capacity to increase transcription of a heterologous promoter in glial and HeLa cells. Using gel-shift and UV-cross linking analysis, we have demonstrated that this region interacts

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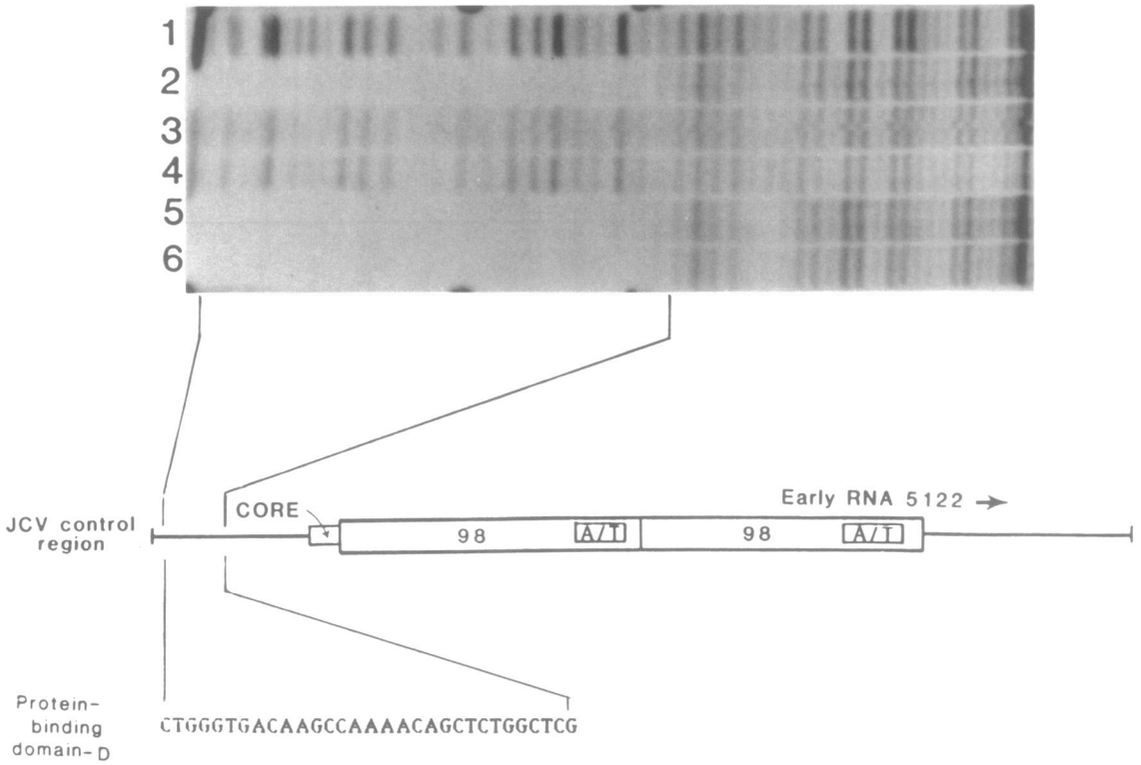

Figure 1. Factor(s) that binds to control region of JCV early gene. DNaseI protection analysis of the JCV sequences with protein derived from glial cells. Reactions contain approximately 10^4 cpm (50 pmol) of ^{32}P 5'-end-labeled DNA probe (290 bp NcoI-HindIII fragment of JCV labeled at the NcoI site) that was incubated at 0°C for 20 min alone (lane 1) or together with protein (lanes 2–6) in the absence of competitor DNAs (lane 2) or in the presence of 100 and 500 ng oligo-D (lanes 3 and 4) or 100 and 500 ng unrelated oligonucleotides, followed by treatment with DNaseI. The map below indicates the location of the binding site with respect to the 98 bp enhancer/promoter, and the sequence composition of the synthetic oligonucleotide-D (Oligo-D).

with nuclear proteins present in glial and HeLa extracts and forms complexes with similar electrophoretic mobilities on SDS-polyacrylamide gels. The possible role of this protein in tissue-specific transcription of JCV_E promoter in brain cells is discussed.

RESULTS

Nuclear proteins derived from glial cells interact with sequences located upstream from the 98bp enhancer regions of JCV

We recently described the identification of several distinct regions within the JCV 98 bp enhancer/promoter region that specifically interacts with protein factors present in human fetal brain nuclear extract (22). DNaseI protection analysis of the JCV control region revealed a pattern of footprinting located 240 nucleotides from the origin of viral DNA replication, approximately 34 nucleotides upstream from the 98 bp repeats, by proteins derived from glial and non-glial L-extracts (23). Here, we have extended our studies and utilized a synthetic double-stranded oligonucleotide to examine the specificity of these interactions and to further investigate transcriptional activity of these protein binding sequences in glial cells. As shown in Figure 1, preincubation of the glial whole cell extract with 0.1 and 0.5 μg of unlabeled oligonucleotide, representing sequences located upstream from the 98 bp repeat (designated domain D), abolished the protection of JCV DNA by glial cell-derived protein (compare lane 2 with 3 and 4). Under identical conditions, preincubation of 0.1 and 0.5 μg of unrelated DNA fragments, representing the upstream regulatory sequences of myelin basic protein promoter at −250 to −289, had no significant effect on footprinting patterns (compare lane 2 with 5 and 6). These results suggest that the protein factor(s) recognized by the JCV DNA has sequence-specific DNA binding properties and perhaps this interaction is independent from the sequences located in the 98bp repeats.

Transcriptional activity of the protein-binding domain 'D' in glial and HeLa cells

Previous studies in our and other laboratories have indicated that the intact JCV early promoter, containing domains A, B, C, and D, is selectively more active in glial than in non-glial HeLa cells (20, 21, 23, 24). Here, we performed *in vivo* and *in vitro* assays using glial and non-glial cells and extracts. To examine the transcriptional activity of protein binding domain-D, we used a transient transfection assay to investigate the role of this region in concert with a simple polymerase II transcription unit. The double-stranded oligonucleotide-D was cloned into the BglII site immediately upstream of the enhancerless SV40 promoter in the $pA_{10}CAT_2$ vector (25). The recombinant clone was detected by dot-blot hybridization and sequenced to determine the number and orientation of the insert. The plasmid containing only one copy in the sense orientation, $pA_{10}CAT_2$-D (Fig. 2A), was selected and introduced into glial and HeLa cells by the ca-phosphate precipitation method (26). Results of the CAT enzymatic assay of $pA_{10}CAT_2$-D revealed approximately 6-fold higher CAT activity compared with the activity from plasmid $pA_{10}CAT_2$ in glial (Fig. 2B, top) and about 3-fold in HeLa cells (Fig. 2B, bottom). Perhaps it should be mentioned that a minor

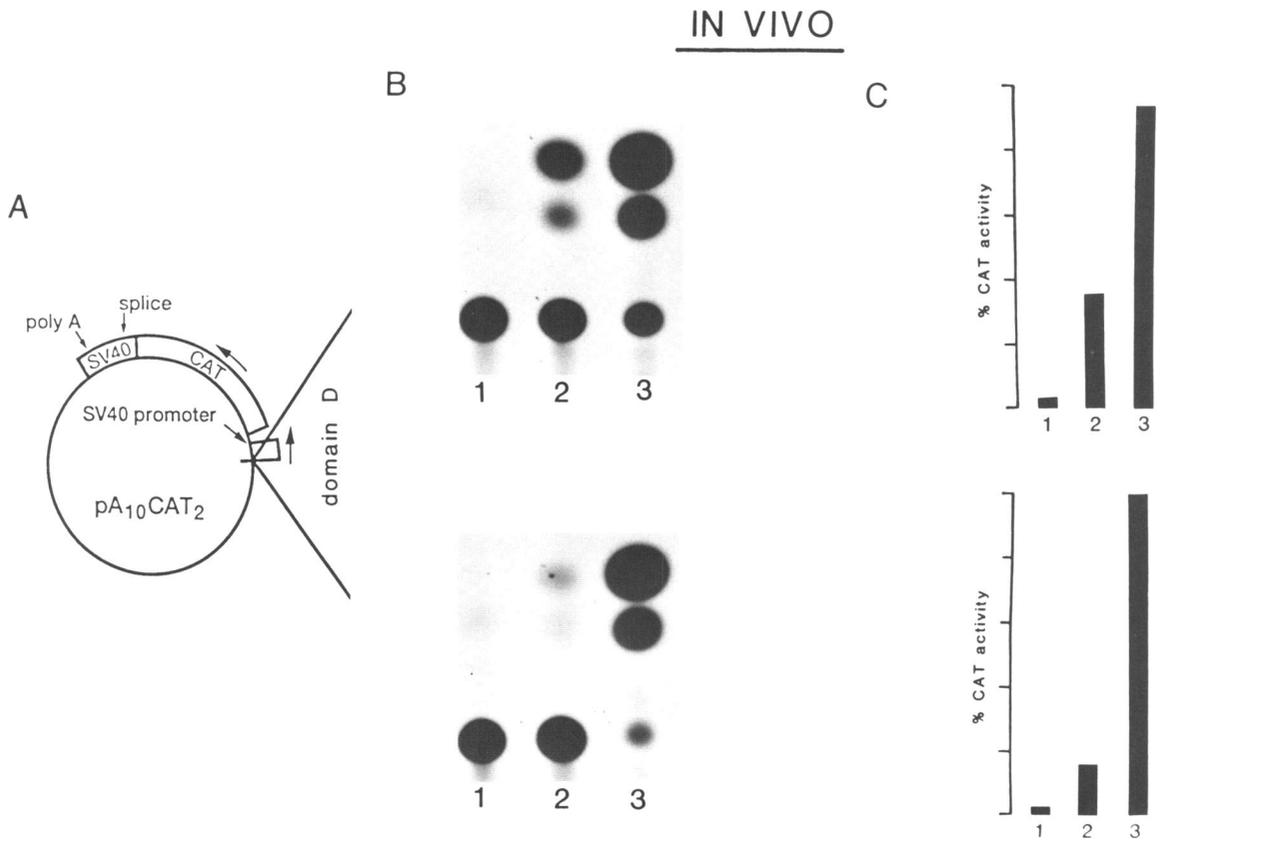

Figure 2. Functional analysis of the protein-binding region in glial and HeLa cells. (A) Synthetic complementary oligonucleotide (oligo-D) homologous to the JCV sequence was inserted at the BglII site of $pA_{10}CAT_2$ which contains the SV40 21-bp repeat that drives CAT gene expression but lacks the enhancer elements (25). (B) 10 μg of supercoiled plasmid DNA was transfected into glial cells (top) and HeLa cells (bottom) using the ca-phosphate precipitation method (26). Cell extracts were prepared at 48 h post-transfection and CAT activity was determined as described by Gorman *et al.* (30). Lane 1, $pA_{10}CAT_2$; Lane 2, $pA_{10}CAT_2$-D; and lane 3, pRSV-CAT. (C) Data represent the level of ^{14}C-chloramphenicol conversion to its acetylated form which were normalized by the level of the β-galactosidase activity produced by the co-transfected pRSV-βgal control (27). Bar 1, $pA_{10}CAT_2$; bar 2, $pA_{10}CAT_2$-D; and bar 3, pRSV-CAT.

activation, if any, was also detected when the D-fragment was cloned in the antisense orientation (23). The control plasmid pRSV-CAT, containing the Rous Sarcoma virus LTR promoter positioned in front of the CAT gene (34), showed comparable CAT activity in both cell types (Fig. 2B, lane 3). CAT activity throughout these experiments was normalized by β-galactosidase activity derived from the co-transfected expression vector pRSV-βgal (27).

Next, we used transcriptionally active whole cell extracts derived from glial and HeLa cells to examine the effect of D-domain on JCV_E promoter activity. The template DNA used in our experiment, pBJC, was digested with the endonuclease SphI 450 bp downstream from the major RNA initiation site (28) and incubated separately with 12 μg of glial and HeLa extracts. As shown in Figure 3A, the glial extract efficiently transcribed JCV_E promoter *in vitro* (lane 1). Interestingly, significant levels of transcription from the JCV_E promoter was also observed in HeLa extract (Fig. 3B, lane 1) indicating that additional factors which are not present in the *in vitro* system, contribute to the specificity of JCV_E promoter activity. We noticed that the level of JCV_E promoter activity in glial extract was consistently higher than in HeLa extracts. To examine the effect of domain-D on the *in vitro* transcription of JCV_E promoter we performed competition experiments in which the D-binding protein(s) was sequestered from the extracts by pre-incubation with 0.1 and 0.5 μg of oligo-D fragment. As a control we applied a similar treatment and used non-related oligonucleotide as described in

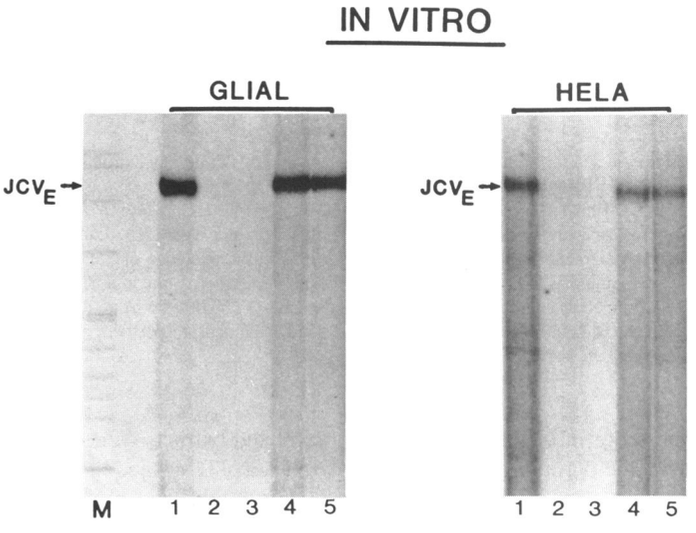

Figure 3. Effects of the protein-binding region D on transcription of the JCV_E promoter *in vitro*. Five hundred nanograms of linearized cloned JCV DNA was transcribed *in vitro* in glial or HeLa extracts in the absence or presence of competitor DNAs. Lane 1, no competitor DNA; Lanes 2 and 3, competition with 100 and 500 ng of oligo-D, respectively; Lanes 4 and 5, *in vitro* transcription of JCV_E when 100 ng and 500 ng of unrelated competitor DNAs, respectively, were added to the extracts. M contained labeled DNA marker (pBR322 MspI digest). The arrow indicates the position of the 450-nucleotide *in vitro* synthesized RNAs.

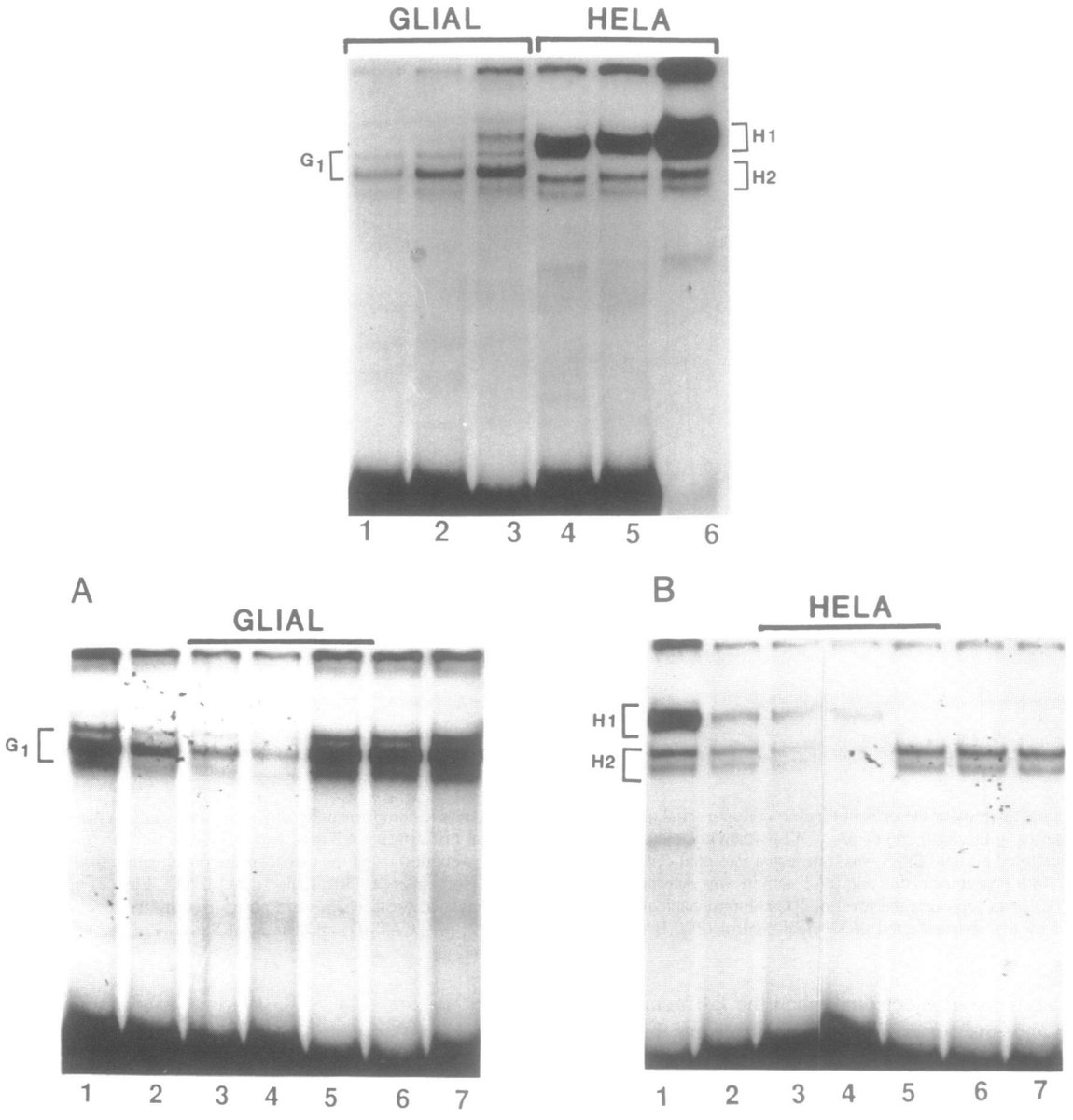

Figure 4. Protein-binding analysis of the D-domain. Whole cell extracts were prepared from glial and HeLa cells as previously described (35). Approximately 20,000 cpm of [^{32}P] end-labeled double-stranded oligonucleotide fragments were incubated in 3–10 μl of nuclear extract (30–100 μg protein) in the presence of 60 mM NaCl, 50 mM Hepes (pH 7.9),12 mM DTT, 4 mM $MgCl_2$, 50 mM Tris (pH 8.0), 0.33 mg/ml poly(dI-dC), and 10% glycerol for 30 min on ice. The resulting complexes were resolved on 9% native polyacrylamide gels (36). Lanes 1 through 3, show the band patterns of oligo-D probe in the presence of 3, 5 and 10 μl of glial extracts; Lanes 4–6, binding reactions in the presence of 3, 5, and 10 μl of non-glial HeLa extract. A, B. Competition analysis of D-binding factors with JCV regulatory D-domain. Binding reactions were carried out in a 30 μl final volume with 30 μg of protein extract derived from glial cells (panel A), or HeLa cells (panel B), with 20 ng of end-labeled probe-D (20,000 cpm) and 5 μg poly(dI-dC). Lane 1, no competitor DNA added; Lanes 2–4, binding reactions in the presence of 100, 500 and 1,000 ng of unlabeled D-fragment, respectively; Lanes 5–7, binding reactions in the presence of 100–1,000 ng of unrelated competitor fragment.

the footprinting experiment. The pretreated extracts were mixed with the optimum amount of linearized pBJC DNA and the RNA product was resolved by a urea-polyacrylamide denaturing gel (29). Addition of 0.1 and 0.5 μg of oligo-D abrogated transcription of JCV_E promoter in glial and HeLa cells (Fig. 3, compare lane 1 with 2 and 3). Preincubation with 0.1 and 0.5 μg of non-related oligonucleotide had a minimal effect on the JCV_E promoter in HeLa extract. This result suggests that HeLa extract may possess an additional factor(s) that is also recognized by the unrelated DNA. These competitors showed no significant effect on transcriptional activity on a control heterologous promoter, i.e. c-myc (Ahmed, unpublished data).

Overall, the above data suggest that the JCV_E promoter, in addition to the proximal elements located in the 98 bp repeat, consists of a distal regulatory sequence, D-domain, that is required for activation of the JCV_E promoter in glial and HeLa cells. The relationship between the D-domain and those located in the 98 bp sequence remains to be determined.

Formation of specific DNA-protein complexes by the D-oligonucleotide

Since the effects of cis-acting regulatory elements are generally known to be mediated by trans-acting cellular factors, we used the band-shift assay to determine whether the D-domain exerts

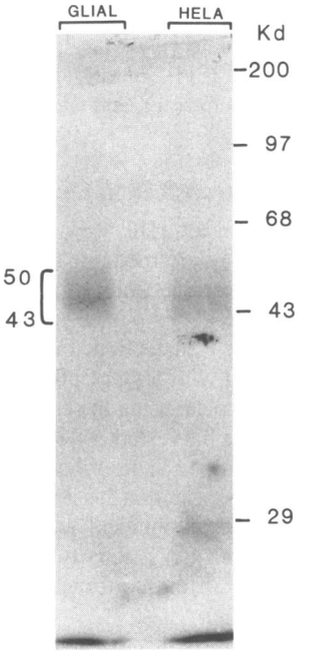

Figure 5. Identification of D-binding protein by UV-cross linking. Binding of BUdR incorporated oligo-D to the proteins and UV-cross linking was performed according to the procedures described in Materials and Methods. Gel-shift bands representing the specific DNA-protein complexes formed in HeLa (i.e. complex H_2) and glial (i.e. complex G_1), were exised from the native gel. Protein-DNA complexes were then eluted and analyzed in a 10% SDS-polyacrylamide gel. The free probe in this gel migrated with a molecular size of less than 10 Kd, shown at the bottom of each lane.

its effect on the *in vitro* transcription of JCV_E promoter by direct binding to nuclear proteins present in the glial and HeLa cells. The double-stranded oligo-D fragment was end-labeled by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and incubated in increasing concentrations of nuclear extract prepared from glial and HeLa cells. In the presence of glial extract, oligo-D formed complexes that were resolved as closely migrating bands designated G1 (Fig. 4, lanes 1–3). In HeLa extracts, two major classes of complexes were observed which migrated slower (H1) and faster (H2) than G1 (Fig. 4, lanes 4–6). When the glial extract was preincubated with 0.1, 0.5 and 1 μg of the unlabeled oligo-D and used as a source of binding protein, the formation of DNA-protein complex with labeled oligo-D was diminished (Fig. 4A, compare lanes 1 and 2–4). The preincubation of the extract with a similar concentration of unrelated competitor DNA fragment showed no significant effect on the intensity of the G1 complex (Fig. 4A, compare lane 1 with 5–7). In HeLa extract, formation of the H1 and H2 complexes was inhibited by the preincubation of protein extract with unlabeled oligo-D. In the presence of the unrelated competitor DNAs, formation of the H1 complex was blocked, whereas the H2 complex association remained unchanged. It is likely that the formation of the H1 complex with the unrelated DNA resulted in reduced transcriptional activity of JCV_E promoter in HeLa extract (Fig. 3B, lanes 4 and 5). The above results indicate that preincubation of glial and HeLa extract with oligo-D effectively depletes specific DNA-binding proteins. Whether or not depletion of the G1 and H1 complexes result in the observed decreased levels of JCV_E promoter activity in an *in vitro* assay remains to be determined.

Identification of D-binding protein by UV-cross linking analysis

To further characterize the protein factor(s) involved in binding the D-domain of the JCV-control region, we performed UV-cross linking experiments using oligo-D as a probe. The internally labeled oligo-D fragment containing BUdR was separately incubated with glial or HeLa extract for 30 min, UV irradiated for 10 min and the nucleoprotein complexes were resolved on a native polyacrylamide gel. A short exposure of the gel was taken to locate the position of the G1 and H1 complexes on the gel which were then excised, eluted, and further resolved by SDS-PAGE. In contrast to the electrophoretic pattern of the G1 and H2 complexes in the native gel, the complexes comigrated as broad bands in the range between 43–50 Kd polypeptides (Fig. 5). This suggests that an additional factor/cofactor may be involved in these complexes. The HeLa-specific complex H1 has a molecular weight of approximately 97 Kd (data not shown). There also appears to be a 29 Kd band present in the HeLa complex, though it was not reproducibly observed. At the present time, we do not know whether the proteins present in complexes G1 and H2 are the same, though they do comigrate on SDS-PAGE and function similarly to stimulate transcription of JCV_E *in vitro*. While the proteins present in these complexes may be identical, it is also possible that they may represent distinct members of a family of transcription factors that recognize a common activator motif. In addition, the 97 Kd protein in the H1 complex also appears to play some role in JCV_E transcription in HeLa cells. The nature of these proteins and their roles as transcriptional activators is currently under investigation.

DISCUSSION

Experiments described here identified a novel activator motif located upstream of the 98 bp enhancer region that increases the transcriptional activity of JCV_E and heterologous promoters in both glial cells and extracts. It is of interest that this distal element, in contrast to the proximal elements in the 98 bp repeat, stimulates the activity of a heterologous promoter in non-glial HeLa cells. This observation suggests that the expression of JCV_E promoter in glial cells is mediated by a set of complex control regions composed of multiple cis-acting elements, each with a varying degree of cell-type specificity. Although the mechanistic details of JCV_E promoters and enhancers are not known, it is likely that these elements activate transcription by communicating with each other through protein-protein interactions and DNA binding. Albeit, this process requires the initial binding of protein factors to the sequences within each module. In previous studies, it has been revealed that the JCV_E promoter contains several protein binding domains within the 98 bp sequence (22). Domain-A contains two copies of CAAT box sequences and interacts with an 82 Kd protein present in both brain and HeLa cells. This domain functions as a transcriptional element *in vivo* and *in vitro*. Domain B, located in the middle of the 98 bp sequence, interacts with a major 45 Kd and a minor 88 Kd protein derived from brain and 85 Kd and 230 Kd proteins from HeLa cells (22). The 45 Kd protein from brain was shown to stimulate the transcription of JCV_E promoter in HeLa extract (24). Domain C, containing a TATA sequence and a tract of adenosines (dA), also stimulates transcriptional activity of JCV_E and heterologous SV40 promoter *in vivo* and *in vitro*, respectively. This domain binds to two proteins of 78 Kd and 80 Kd from brain and HeLa cells. Most recently, we have identified a novel functional region

between Domain B and Domain C designated OP_I, that positively and negatively regulates JCV_E and JCV_L promoters. This OP_I region binds to a 56 Kd and a 35 Kd protein. Here, our binding studies indicate that the distal element, Domain-D, interacts specifically with protein found in both glial and HeLa cells. Unlike the proteins which recognize Domain B (24), Domain D binds to proteins derived from both HeLa and glial extracts with similar electrophoretic mobilities on SDS-polyacrylamide gels. Based on the electrophoretic mobility of the bound protein(s) that exhibited no significant cell-dependent diversity in glial and HeLa extract, and the activity of this element in both cell types, it is likely that this upstream domain is recognized by similar activators expressed in various cell types. Interestingly, in contrast to the intact JCV regulatory region, the D-element of JCV is not a classical enhancer and functions only in the sense orientation, i.e. toward the JCV early gene. It should be mentioned that in the anti sense orientation, the D-domain resides in the leader of the viral late RNAs. Notably, Domain-D of JCV_E promoter contains a 15-bp sequence with striking homology to the sequences present in the BK virus enhancer/promoter region (19). At present, it is not known whether the D-domain contains one or multiple activator elements. The use of methylation interference technique and site-specific mutagenesis should help to answer these questions (in progress).

A common feature of many viral and cellular promoters and enhancers is that they are composed of multiple functional modules each with a different degree of activity. These modules may be generic activators which are shared in common with a number of genes with a universal expression pattern, or may be specific activators which restrict expression of the promoter to certain cells or stages. Although, in many genes the overall organization of these modules is similar, the combination of them may contribute to the specificity of the promoter/enhancer. Our analysis of the human glial specific viral promoter, JCV_E, has revealed the presence of several protein binding domains with a different degree of activity in glial and non-glial cells. However, our data has not answered whether or not these elements and their binding proteins play a functional role in JCV_E promoter activity in the lytically infected glial cells. The answer to this question warrants more detailed and direct structural-functional analyses using mutant viruses. Studies are in progress to further characterize the JCV_E promoter elements, purify the protein factors that recognize these elements, and assess their ability to stimulate JCV_E transcription *in vitro* in glial and non-glial contexts.

MATERIALS AND METHODS

Plasmids, oligonucleotides and labeled fragments

The plasmid pBJC was made by inserting the BamHI digested JCV DNA into pBR322. The plasmid $pA_{10}CAT_2$ is an enhancerless SV40-CAT construct (25) and was used as a vector to construct $pA_{10}CAT_2$-D. Plasmids pRSV-βgal and pRSV-CAT contain the RSV-LTR promoter in front of the β-galactosidase and CAT genes, respectively (30, 34). Oligonucleotides were synthesized by the phosphoramidite method on an automated synthesizer (Applied Biosystem, Inc.). The nucleotide composition of the oligonucleotide-D was copied from the JCV gene between nucleotides 240 to 268 with respect to the origin of replication (19). Heterologous (unrelated) oligonucleotide DNA used in competition assays was derived from the myelin basic protein gene between -250 to -289 (31). Radiolabeled double-stranded oligonucleotide probes were prepared by 5'-end labeling using T4-polynucleotide kinase, and by nick-translation in the presence of [^{32}P]dCTP, BUdR, dATP, dGTP and DNA polymerase I (Kornberg enzyme), and were purified on 9% native polyacrylamide gels.

Tissue culture, DNA transfection and CAT assay

Hamster fetal glial cells, HJC (31), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were plated at a density of approximately $2-3\times10^6$ cells per 60-mm dish and refed with fresh medium 4 h before transfection. Five to 10 μg of supercoil test plasmid DNA along with 5 μg of pRSV-βgal plasmid DNA (30) was transfected per plate using the ca-phosphate precipitation method (26). Cells were harvested at 48 h post-transfection and CAT activity was determined as described by Gorman *et al.* (30). Each transfection was repeated at least three times and percent conversion of (^{14}C)-chloramphenicol was determined by liquid scintillation counting and CAT activity was normalized to the β-galactosidase activity in the co-transfected cells.

Extract preparations and *in vitro* transcription

Whole cell extracts were prepared from HJC and HeLa cells as described by Manley *et al.* (32). Transcription mixtures (50 μl each) contained 12 mM Tris (pH 7.9), 12% glycerol, 60 mM KCl, 2 mM $MgCl_2$, 0.6 mM CTP, 0.6 mM ATP, 0.6 mM GTP, 0.5 mM [^{32}P]UTP, 0.5 μg of template DNA, and 3 to 5 μl of whole cell extract (approximately 50 μg of protein). In competition assays, extracts were preincubated with the competitors at 0°C for 20 min prior to adding the template DNAs. After incubation for 60 min at 30°C, run-off transcripts were analyzed on a denaturing 6% polyacrylamide gel as described by Maxam and Gilbert (29). DNA template was obtained by digestion of plasmid pBJC with SphI to generate the 450 nt of the run-off transcript.

DNA-protein interaction techniques

DNAse 1 footprinting was done by incubating 50 μg of extract prepared from glial cells with 2 μg of pUC18 DNA on ice for 20 min followed by the addition of 30,000 cpm end-labeled DNA fragment (labeled at NcoI site, map position 285). The reaction was continued for an additional 30 min and then transferred to 22°C (room temperature) for DNase treatment. One μl of freshly diluted DNaseI (5 μg/ml) was added to each reaction and allowed to proceed for 3 min. The reaction was stopped by the addition of 2 vol of stop buffer (20 mM EDTA, 20 mM Tris-HCl, 0.2% SDS, and 50 μg of tRNA per ml). After phenol extraction, DNA was analyzed on a polyacrylamide-7M urea gel (29). Band-shift assays were done as described (22, 33) with several modifications. Protein samples were incubated with 200–500 ng/ml (5000 cpm/ng) of end-labeled double-stranded oligonucleotides in the presence of 300 μg/ml poly[dI-dC] (Pharmacia) in a final volume of 30 μl. Incubations were carried out for 30 min at 0°C and samples were fractionated on low ionic strength 9% polyacrylamide gels for 3 h at 185V in $0.3-0.5\times$TBE at 4°C. Gels were dried on Whatman 3 MM paper and bands detected by autoradiography. UV cross-linking was performed by incubating BUdR incorporated labeled DNAs with protein extracts as described above. After incubation, reaction mixtures were exposed to UV light (302 nm) for 10 min, resolved by low-ionic gel electrophoresis. The complexes were excised from the gels and resolved on the SDS-polyacrylamide gel. The molecular

sizes of the complexes were compared to pre-stained high molecular weight protein standards obtained from Bethesda Research Laboratories.

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