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Cell Type-Specific Expression of JC Virus Early Promoter Is Determined by Positive and Negative Regulation†

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We analyzed control sequences of the human papovavirus JC virus (JCV) to define the *cis*-acting elements that regulate specific expression of the viral early region genes in glial cells. Nuclear run-on transcription, S1 analysis, and chloramphenicol acetyltransferase enzyme activity in a transient transfection assay established that the cell type-specific expression of JCV early genes is determined at the transcriptional level. Using DNase footprinting analysis of nuclear proteins prepared from glial and nonglial cells, we located four regions within the JCV control sequences that specifically interacted with the proteins. In glial cells, all four domains contributed to the specific expression of a heterologous promoter, whereas in nonglial cells, two protein-binding regions showed no effect on basal transcriptional activity and the other two domains significantly downregulated transcription of the promoter. We conclude that cell type-specific transcription of the JCV early promoter is under both positive and negative regulation in eucaryotic cells.

The human papovavirus, JC virus (JCV), has been repeatedly isolated from demyelinated plaques in the brains of patients with progressive multifocal leukoencephalopathy and is thought to be the etiological agent of this disease (9, 19–22). JCV exhibits a defined tissue tropism for glial cells. In tissue culture, this virus replicates efficiently only in human fetal glial cells (19). Studies by Kenney et al. (10) have demonstrated that the JCV control region possesses sequences that are important for cell type-specific expression of the viral early proteins in human fetal glial cells. Unlike the tissue specificity of the early-gene activity, viral DNA replication occurs in any primate cell type when the viral early protein, T antigen, is provided (2). Thus, the host range restriction of this virus can be determined on at least two levels, cell type-specific transcription of early RNA synthesis and species-specific replication of the viral DNA in primate cells.

The control region of the JCV genome contains two 98-base-pair (bp) tandem repeats located on the late side of the origin of DNA replication (4). There is a 15-nucleotide, AT-rich segment within each of the two 98-bp repeats and one 8-nucleotide segment, GTGGAAAG, homologous to the simian virus 40 enhancer core sequence, on the late side of the 98-bp repeat region. We have recently shown that the 98-bp enhancer region contains at least three protein-binding domains, each of which interacts with a distinct nuclear protein from HeLa and human fetal brain extracts (K. Khalili, J. Rappaport, and G. Khoury, EMBO J., in press). In this study, we used synthetic oligonucleotides spanning the 98-bp sequence and its flanking sequences to examine the role of each protein-binding domain in the cell type-specific expression of the JCV promoter. We found that, in addition to positive transcriptional regulation of the viral promoter in glial cells, there are sequences within the viral genome that downregulate transcriptional activity of the early promoter in nonglial cells.

To analyze the activity of the JCV early promoter, two constructs, each containing the JCV control region with either one or two copies of the 98-bp sequences located upstream of the chloramphenicol acetyltransferase (CAT) gene (10, 12), were introduced into a wide variety of glial and nonglial cells by the calcium phosphate procedure, and production of CAT enzyme, which is dependent on transcription governed by JCV regulatory sequences, was quantitated. Virtually no CAT activity was detected in nonglial cells (Fig. 1, lanes 1 through 4), whereas substantial activity was detected in all glial cells of different species (Fig. 1, lanes 6 through 9). In addition, expression of CAT in a construct with only one copy of the 98-bp repeat indicated that this one copy is sufficient to activate the JCV early region in glial cells (Fig. 1, lanes 12 and 13). The level of CAT mRNA initiated from the JCV early promoter was also measured by using S1 protection of total cellular RNA prepared from lysates of glial and nonglial cells. For a probe, we used a 485-nucleotide uniformly labeled single-stranded construct derived from an m13 vector containing a portion of the CAT coding sequence (11). Figure 2A demonstrates the protection of a 256-nucleotide DNA fragment corresponding to complete protection of the CAT sequences within the probe by CAT mRNA produced in glial cells. This transcript was undetectable in nonglial cells.

To determine whether the level of CAT mRNA reflects the rate of transcription and not the steady-state level of mRNA, we performed nuclear run-on transcription experiments using nuclei isolated at 48 h after transfection. This assay focuses on the elongation step as a measure of the rate of RNA synthesis (8, 18). The adenovirus VA1 gene (3) was cotransfected with the JCV-CAT chimeric plasmid and used as an internal control to ensure comparable transfection efficiencies in these experiments. The ^{32}P-labeled RNAs synthesized in cell-free nuclei were hybridized to CAT (Fig. 2B) and virus-associated (VA) (Fig. 2C) sequences by the slot-blot technique (Schleicher & Schuell, Inc.). Figure 2 shows the levels of newly transcribed CAT mRNA and VA RNA. Again, no CAT RNA was detected in nonglial cells, whereas high levels of CAT RNA were synthesized in glial cells. The rate of VA RNA synthesis in both cell types

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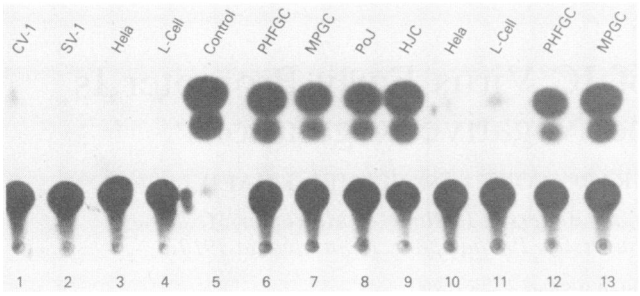

FIG. 1. Transcriptional activity of the JCV enhancer-promoter in glial and nonglial cells. A 15-μg sample of pBJC-CAT (10) containing JCV early promoter with two copies of the 98-bp repeat was introduced into monkey kidney cells (CV-1), human fetal kidney cells (SV-1) (13), human cervical carcinoma cells (Hela), and mouse fibroblasts (L-Cell) (lanes 1 through 4), primary human fetal glial cells (PHFGC), mouse primary glial cells (MPGC), human fetal glial cells (PoJ) (14), and hamster glial cells (HJC) (5, 22) (lanes 6 through 9) by the calcium phosphate technique (7). pBJC-CAT-98 containing JCV early promoter with only one copy of the 98-bp repeat was introduced into HeLa and L cells (lanes 10 and 11) and into primary human fetal glial cells and mouse primary glial cells (lanes 12 and 13). Cell extracts were prepared 48 h posttransfection, and CAT activity was determined (6). Lane 5 represents chloramphenicol treated with a commercially obtained CAT enzyme (control).

remained approximately constant. Together, these results establish that cell type-specific expression of JCV early genes is primarily regulated at the transcriptional level, as indicated by the rate of transcription, stability of the message, and amount of translation product (CAT activity).

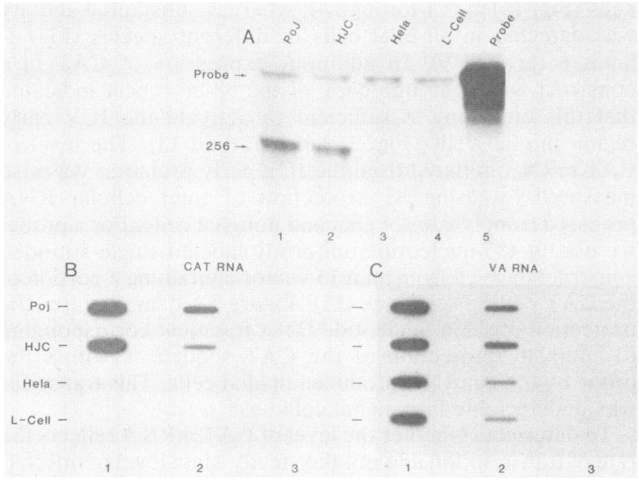

FIG. 2. CAT RNA analysis by S1 protection and nuclear run-on assays. (A) S1 nuclease analysis of CAT RNA obtained from transfected glial and nonglial cells. At 48 h posttransfection, total RNAs were isolated from the cells and hybridized to a single-stranded uniformly labeled CAT DNA probe (11). Hybridization and S1 digestion were performed as previously described (15). Lanes: 1 and 2, RNA of transfected PoJ and HJC cells, respectively; 3 and 4, HeLa and L-cell RNA, respectively; 5, control S1 probe. (B and C) Analysis of newly synthesized RNA by run-on nuclear assay. At 48 h posttransfection, cells were harvested and nuclei were prepared for run-on transcription assay (8, 18). Nuclei were allowed to elongate RNA chains in vitro, and acid-precipitable ^{32}P-labeled nuclear RNA was isolated and hybridized with 0, 5.05, and 0.05 μg (lanes 1 through 3, respectively) of denatured CAT DNA (B) or VA DNA (C) as the probe by slot-blot hybridization.

The action of *cis*-regulatory elements (enhancer-promoter) is mediated, in general, by their interaction with *trans*-acting cellular proteins (16). To identify these elements within the JCV control region, we first used DNase I footprinting to locate the protein-binding domains of the JCV control region. As a probe, we used a 295-nucleotide DNA fragment 5′-end-labeled position (map position 285) that contains two copies of the 98-bp repeat and 130 nucleotides of the sequences upstream of the repeat at the viral DNA late region. The fragment was incubated with equal amounts of protein from nonglial cells (L cells) or glial cells (HJC) and partially digested with DNase I. The cleavage products were then analyzed by electrophoresis in denaturing polyacryl-

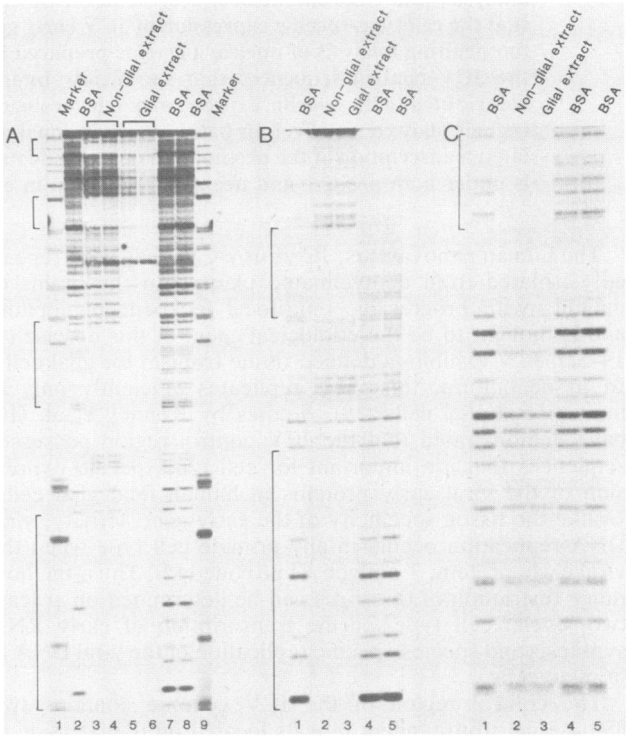

FIG. 3. Targeting of the protein-binding domains of the JCV enhancer-promoter sequences by DNase I footprinting. DNase I footprinting reactions were carried out as follows. A 20-μg sample of protein prepared from L-cell and HJC nuclei by the procedure described by Dignam et al. (1) was preincubated with 2 μg of pBR322 on ice for 20 min, and 3 μl of end-labeled DNA fragment (10,000 cpm in 10 mM Tris [pH 8.0]–0.1 mM EDTA) was added to the sample. The reaction was kept on ice for 30 min and then at 22°C for 5 min. Finally, 1 μl of freshly diluted DNase I (5 μg/ml) (Sigma Chemical Co.) was added, and the reaction was allowed to proceed for 1 to 3 min. The reaction was stopped by the addition of 1 volume of 20 mM EDTA–20 mM Tris hydrochloride (pH 8.0)–0.2% sodium dodecyl sulfate and 50 μg of tRNA per ml. After phenol extraction, DNA was precipitated, denatured, and analyzed on a polyacrylamide–7 M urea gel (17). BSA, Bovine serum albumin. (A) A 295-nucleotide DNA fragment labeled at NCO-1 site (map position 285) was used as a probe. (B and C) Two smaller DNA fragments, *Pvu*II-*Sst*I and *Hin*dIII-*Sst*I, respectively, located within the JCV control region were used as probes. Brackets indicate regions representing protein-binding domains. From the top of panel A, first and second brackets represent the central region of each 98-bp repeat (domain B), and third and fourth brackets represent domains A and D (see Fig. 4 for detail). From the top of panel B, first and second brackets correspond to domains A and D. In panel C, the bracket represents domain B in the center of the 98-bp sequence.

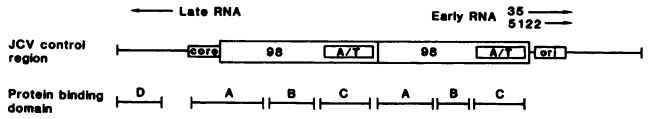

FIG. 4. Structure of JCV control sequences and the protein-binding regions within. The AT box within each 98-bp repeat (98) contains a 5′-TATATATAAAAAAAA-3′ sequence possibly analogous to the Goldberg-Hogness box. The *ori* segment represents the 20-bp origin of DNA replication. The core segment indicates the location of an octameric motif, the 5′-GTGCAAAG-3′ simian virus 40 core sequence. Locations are based on our present and previous studies (Khalili et al., in press). Domain B corresponds to the first and second brackets of Fig. 3A, and domains A and D overlap with the protected region indicated by the third and fourth brackets of Fig. 3A.

amide gels. Four distinct protected areas were observed in glial and nonglial cell extracts (Fig. 3A, brackets). Two were located in the center of each 98-bp sequence, one at the 5′ region flanking the 98-bp repeat on the late side of the DNA, and one further upstream at the late translational initiation site. Figure 3B and C show the footprinting of the smaller fragments of the JCV control regions. No significant differences were observed in the footprints of the HJC and L-cell nuclear extracts, suggesting that glial and nonglial nuclear proteins recognize similar domains within the viral control sequences. Using gel mobility shift and UV cross-linking assays, we previously demonstrated that two proteins of 82 and 78 to 80 kilodaltons also interact specifically with the 5′- and 3′-terminal regions, respectively, of the 98-bp repeat sequence (Khalili et al., in press). The electrophoretic mobilities of these proteins were similar in glial (brain) and nonglial (HeLa) nuclear extracts. Our data indicate that multiple DNA sequences within the JCV regulatory region bind cellular factors. Figure 4 illustrates the organization of the JCV control region and the protein-binding domains across the viral enhancer-promoter sequences.

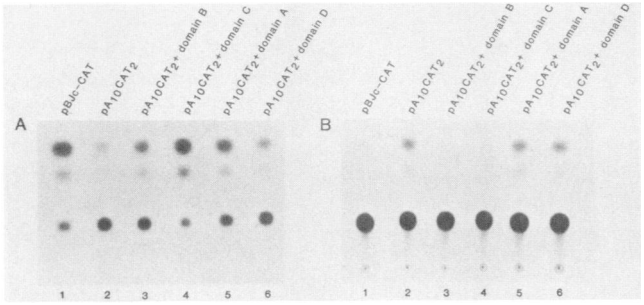

FIG. 5. Role of the JCV protein-binding DNA sequences in cell type-specific transcription of the viral genome. Oligonucleotides spanning the protein-binding sequences, as defined in the legend to Fig. 3, were inserted at the *Bgl*II site of the $pA_{10}CAT_2$ plasmid (12). Nucleotide sequences of these oligonucleotides are as follows: domain A, GGAACCGACGAAAGGTGAAGGGGAACACGAAA; domain B, CTCGAGTACGACCGACCGTCGGTAGGGA; domain C, AAGGGAAAAAAAATATATATGTC; domain D, CCACTGTT CGGTTTTGTCGAGACCGAGC. The orientations of these oligonucleotides in the recombinant plasmid with respect to their locations in the JCV genome were determined by direct sequencing of the DNA. Except for those in domain D, they were all in the early orientation. Each plasmid was introduced into HJC (A) or L (B) cells by the calcium phosphate procedure (7). At 48 h posttransfection, extracts were prepared from the glial cells and CAT enzyme activity was determined.

To analyze the role of each protein-binding domain in regulating JCV promoter activity in glial cells, a series of synthetic DNA fragments representing the protein-binding domains (Fig. 4) was prepared and separately cloned into the $pA_{10}CAT_2$ vector (12) at a position in front of the simian virus 40 promoter. Each construct was then introduced into the glial cells by the calcium phosphate procedure, and production of CAT enzyme was measured after 48 h. CAT production was increased three-, two-, and fivefold in constructs with protein-binding regions A, B, and C, compared with the rate in plasmid $pA_{10}CAT_2$ (Fig. 5A). No significant difference was obtained in $pA_{10}CAT_2$ constructs with region D (map positions 116 to 133). The levels of [^{14}C]chloramphenicol conversions to acetylated forms were significantly higher in pBJC-CAT than in $pA_{10}CAT_2$ vectors (more than eightfold). These results suggest that JCV enhancer-promoter sequences consist of multiple *cis*-acting elements that contribute to transcription of viral early genes in glial cells. In parallel experiments to examine the possible role of each domain in nonglial cells, these constructs were introduced into L cells and CAT activity was assayed at 48 h posttransfection. A basal CAT activity due to the expression of the CAT gene by the simian virus 40 promoter remained intact when domains A and D were independently introduced at the upstream region of the CAT gene (Fig. 5B, lanes 5 and 6). However, introduction of domains C and D abolished the basal level of CAT activity in the cell (Fig. 5B, lanes 3 and 4). These results indicate that the cell type-specific transcription of JCV early promoter is governed by at least two types of overlapping *cis*-acting elements located in the viral control region, sequences that potentiate expression of the viral promoter in glial cells and regions that suppress transcription of the early promoter in nonglial cells. These initial observations leave open the question of whether the proteins recognizing these sequences are the same or different in permissive and nonpermissive cells. Our earlier observations in which the DNA-binding proteins of HeLa and human fetal brain nuclear extracts were compared for their potential to interact with the JCV 98-bp sequences revealed that two of three regions within the 98-bp sequences interacted with similar-size HeLa and brain proteins (Khalili et al., in press). However, the central regions of the repeat recognize two distinct proteins. The simplest conclusion from these results is that the central region of JCV is recognized by multiple protein factors present in different glial and nonglial cells. Which DNA-binding factors actually possess functional activities that lead to the transcription of the viral promoter remains to be investigated.

We have shown here that cell type-specific gene expression of JCV is determined exclusively at the level of transcription and not by stability of the message or translation of mRNA. Moreover, independent of species, all primary and established glial cells support transcription of the viral promoter. Our results also demonstrated that a single copy of the 98-bp enhancer-promoter sequence is sufficient, albeit with the other DNA control sequences, to provide cell-type specificity and that the cell type-specific transcription of JCV is under dual regulation, positive and negative, in eucaryotic cells.

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