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Transcription of a Human Neurotropic Virus Promoter in Glial Cells: Effect of YB-1 on Expression of the JC Virus Late Gene

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We have isolated a partial recombinant cDNA clone from a HeLa expression library which encodes a protein capable of binding to the central region of the human neurotropic JC virus (JCV) enhancer/promoter, termed the B region. Sequence analysis revealed a complete homology of the partial cDNA clone to the N-terminal region of a previously described DNA-binding protein, termed YB-1. Band shift analyses have indicated that the bacterially produced YB-1 interacts specifically with the double-stranded B oligonucleotide as well as the corresponding single-stranded DNA fragment representing the early promoter sequence. Further analysis indicated that the YB-1 protein binds specifically to the C/T-rich sequence of the B domain, which is located in close proximity to the TATA box within the virus enhancer/promoter. Results from cotransfection experiments demonstrated that the full-length (YB-1) but not the partial cDNA enhances expression of the JCV late (JCV_L) promoter in glial cells. Cointroduction into glial cells of a recombinant expressing the YB-1 and JCV_L deletion mutants indicated that removal of the C/T-rich sequence of the B domain reduces the level of activation of the virus promoter by YB-1. Further cotransfection experiments revealed that the virus transactivating protein T antigen appears to diminish the ability of YB-1 to activate JCV_L gene expression. RNA studies indicated that YB-1 is expressed in several cell types and tissues. Examination of YB-1 RNA from mouse brain at various stages of development revealed high levels of YB-1 RNA at early stages of development and lower levels at all subsequent developmental stages.

Papovaviruses comprise a large and important family of animal pathogens that are widely distributed in nature and produce latent and chronic infection in their natural hosts (37). Infection with the human neurotropic papovavirus, JC virus (JCV), is very common and occurs in childhood (31). This virus is an opportunistic pathogen and the etiological agent of progressive multifocal leukoencephalopathy, a subacute demyelinating disease of the central nervous system in patients with defects in cell-mediated immunity (for review, see reference 26). JCV has been shown to replicate exclusively in glial cells, the myelin-producing cells in the central nervous system. The structural organization of this virus is similar to those of two other polyomaviruses, simian virus 40 and BK virus of humans (14). The greatest degree of divergence between these viruses is in the transcriptional control region, which in JCV contains the origin of DNA replication, promoters for early (JCV_E) and late (JCV_L) gene transcription, and tandem 98-bp direct repeats. Several lines of evidence have demonstrated that the transcriptional control region of JCV determines its exclusive neurotropic expression (13, 20, 38). Our laboratory has initiated a detailed examination of cellular factors that restrict JCV_E gene expression to glial cells. Our initial results have suggested that the JCV control region contains several domains that interact with distinct factors differentially expressed in glial and nonglial cells (22). One of these domains, termed the B region, spans the central part of the 98-bp repeats and has been extensively studied by us and others (1, 2). We had previously screened a human brain library and isolated a recombinant cDNA named glial factor 1, which encodes a protein that binds to the B region of the JCV enhancer sequence and has the ability to activate transcription of both the JCV_E and JCV_L promoters (21). In the present study, we have screened a λgt11 library derived from HeLa cells in order to identify common or regulatory factors that may participate in the basal activity of the JCV_L promoter by binding to JCV control sequences. From this search, a partial cDNA, termed HeLa factor 1 (HF-1), corresponding to a previously identified gene termed YB-1 (11) has been isolated.

YB-1 is a member of a gene family, which was initially isolated from a human B-cell expression library by virtue of its capacity to interact with the Y box region, with the sequence CTGATTGG(C/T)(C/T)AA, found within the promoter of class II major histocompatibility complex genes (11). This region is essential for basal as well induced levels of expression of class II major histocompatibility complex genes and several other eukaryotic genes (4, 6, 7, 12, 19). The presence of a CCAAT motif within the Y box of the class II major histocompatibility complex gene and many other YB-1 genes (26, 30, 34, 35, 40) has led to the early assumption that the YB-1 family, like C/EBP (24) and CTF/NF-1 (28, 36), belongs to a class of CCAAT box binding proteins. However, several later studies have reported virtually no association of C/EBP with the YB-1 protein (10, 17, 23, 44). The binding of a recombinant YB-1 cDNA to a pyrimidine-rich oligonucleotide that can adopt an intramolecular triplex, single-stranded structure implies that topological configuration of the DNA molecule may play an important role in determining YB-1 binding to DNA (23).

Other studies have suggested that YB-1 may indirectly participate in translation of RNAs (15, 18, 33, 39). The counterparts of YB-1 in *Xenopus* spp., FRGY1 and FRGY2, are mRNA-binding proteins participating in the storage of maternal mRNAs as nontranslated ribonucleoprotein complexes (29). FRGY2 is localized predominantly in the cyto-

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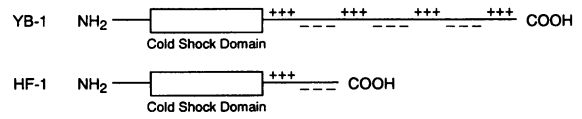

FIG. 1. Comparison of the structures of HF-1 and YB-1. Schematic representation of the full-length YB-1 cDNA and its various functional domains conserved among members of the YB-1 family are noted. The cold shock domain is approximately 80 amino acids and is highly conserved among all members of the family. The arginine/proline-rich domains (+++) and the alternating acidic domains (−−−) are noted. HF-1 includes the cold shock domain and the first basic/acidic cluster of YB-1.

plasm of *Xenopus* oocytes. In somatic cells, FRGY2 increases transcription of promoters containing a Y box and represses translation of mRNA transcribed from promoters containing a Y box (33). Both of these effects are reduced when the Y box is mutated. Altogether, these data suggest that YB-1 has a dual function in both transcription and translation. The Y box may serve as a tether for YB-1 to increase transcription from the promoter and bind to nascent RNA molecules in order to influence their transport to the cytoplasm and subsequent translation.

A comparison of the primary structures of YB-1 from *Escherichia coli*, rat, mouse, frog, and human cells has revealed a high degree of sequence conservation, suggesting an essential functional role in the cell (reviewed in references 42 and 43). Amino acid sequence analysis of YB-1 proteins has revealed a variable N terminus; a central domain, termed the cold shock domain (39); and several clusters of alternating acidic and basic residues in the C terminus (30). The cold shock domain is highly conserved and contains the nucleic acid recognition domain.

In this report, we demonstrate that the YB-1 protein is able to bind to the JCV_L promoter sequence and increase transcriptional activity of the virus promoter in the absence of the early transactivation protein T antigen in glial cells.

Isolation of a cDNA encoding a B region-binding protein from HeLa cells. To study the possible function of the B region-binding protein from nonglial cells in regulation of JCV gene expression, a λgt11 cDNA expression library prepared from HeLa cell RNAs was screened with a probe representing the B region by using the in situ filter detection method (41). Screening of more than 2.5×10^5 plaques resulted in the isolation of a recombinant phage encoding a B region-binding protein, termed HF-1. Sequence analysis of HF-1 revealed a 660-bp DNA fragment with perfect sequence identity to YB-1 (11) and 98% homology to another DNA-binding protein, named dbpB (35). HF-1 contains an open reading frame along its entire length and corresponds to sequences spanning the entire N-terminal region through the cold shock domain and one of the basic/acidic clusters of YB-1 (Fig. 1). Members of the YB-1 family are highly conserved in the cold shock domain, which extends over approximately 80 amino acids. The sequence identity of HF-1 to YB-1 is likely to reflect the isolation of the same gene, or HF-1 is a partial-length clone of YB-1.

Specificity of the nucleoprotein complex formation. The bacterial expression vector pMAL-cRI, which allows synthesis of fusion proteins between maltose-binding protein and cDNA-encoded proteins, was used to study the DNA-binding properties of the proteins encoded by the partial-length (HF-1) and full-length (YB-1) cDNAs. The binding activities of crude bacterial extract, the purified fusion protein, and the purified protein were tested by Southwestern (DNA-protein) analysis and mobility shift assays. Both YB-1 and HF-1 were capable of binding the double-stranded B oligonucleotide DNA, either as purified proteins or as fusion proteins (data not shown). The ability of HF-1 to bind DNA is not surprising, because it contains the cold shock domain thought to be the nucleic acid recognition domain.

To assess the DNA structural and sequence requirements for interaction with the bacterially produced YB-1 protein, competition experiments were performed with double-stranded oligonucleotides representing various regions of the JCV regulatory sequences, as schematized in Fig. 2A, with their sequences detailed in Fig. 2B. These regions were selected on the basis of results from previous studies implicating their importance in basal and/or induced levels of virus early and late gene transcription (2, 9, 21, 32, 38). Unlabeled competitor DNAs were preincubated with purified MBP-YB-1 fusion protein prior to addition of the probe. Oligonucleotides derived from sequences spanning the NF-κB domain (KB), the origin of DNA replication (UP-1), the TATA box (TATA), and an initiator-like sequence (INIT) have no effect on the association of YB-1 with the B oligonucleotide at 25 ng of unlabeled competitors, which represents approximately 25-fold excess of competitor over probe (Fig. 2C, compare lane 2 with lanes 3, 5, 7, and 11). However, addition of 50 ng of the KB and UP-1 oligonucleotides (representing a 50-fold excess) to the reaction showed a minor effect on the binding of YB-1 to the B oligonucleotide DNA probe (Fig. 2C, compare lane 2 with lanes 4 and 6). As expected, 25- and 50-fold molar excesses of the homologous double-stranded unlabeled B oligonucleotide competitor completely abrogated binding of YB-1 to the probe (Fig. 2C, compare lane 2 with lanes 9 and 10). For more precise determination of the YB-1 binding site, two smaller nonoverlapping competitors, B-1 and B-2, contained within the B region were used as the competitors in the band shift assay. The B-1 oligonucleotide contains the C/T-rich region of the B domain, whereas the B-2 oligonucleotide encompasses the consensus NF-1 binding site. As shown in Fig. 2D, inclusion of the B-1 but not the B-2 oligonucleotide in the binding reaction abolished binding of YB-1 to the B oligonucleotide probe (Fig. 2D, compare lane 2 with lanes 3 to 6).

Interestingly, unlabeled single-stranded oligonucleotides comprising the early ($B\text{-}1_E$) but not the late ($B\text{-}1_L$) orientation also efficiently diminish YB-1 binding to the double-stranded B region (Fig. 2D, compare lane 2 with lanes 7 and 8 and lanes 9 and 10). The B-2 sequences in either orientation were unable to inhibit YB-1 binding. Thus, YB-1 is capable of binding to the single- and double-stranded forms of the DNA derived from the C/T-rich region of the B motif.

Furthermore, in vitro binding studies with a truncated YB-1 protein, i.e., HF-1, have indicated a binding profile of the bacterially produced protein similar to those of the single- or double-stranded DNA competitors derived from the JCV regulatory region (data not shown).

Effect of YB-1 on expression of JCV promoter. To assess the effect of YB-1 and its truncated form, HF-1, on transcription of the JCV_L promoter, a reporter plasmid ($pJCV_L$-CAT) expressing chloramphenicol acetyltransferase (CAT) under direction of the JCV_L promoter was introduced into human glial cells alone or with plasmids expressing YB-1 or HF-1 under the cytomegalovirus promoter. We observed that YB-1 enhances transcription from the JCV_L promoter in transfected glial cells, which is most clearly seen at higher concentrations of YB-1 expressor plasmid (Fig. 3A, compare lane 1 with lanes 2 and 4). Under identical conditions, expression of HF-1 in transfected glial cells exhibited no stimulatory effect on virus promoter activity (Fig. 3B). In fact, we noticed that overexpression of HF-1 in transfected cells slightly reduced the level of the virus gene expression. These results suggest that YB-1 has the capacity to enhance expression from the virus late promoter in

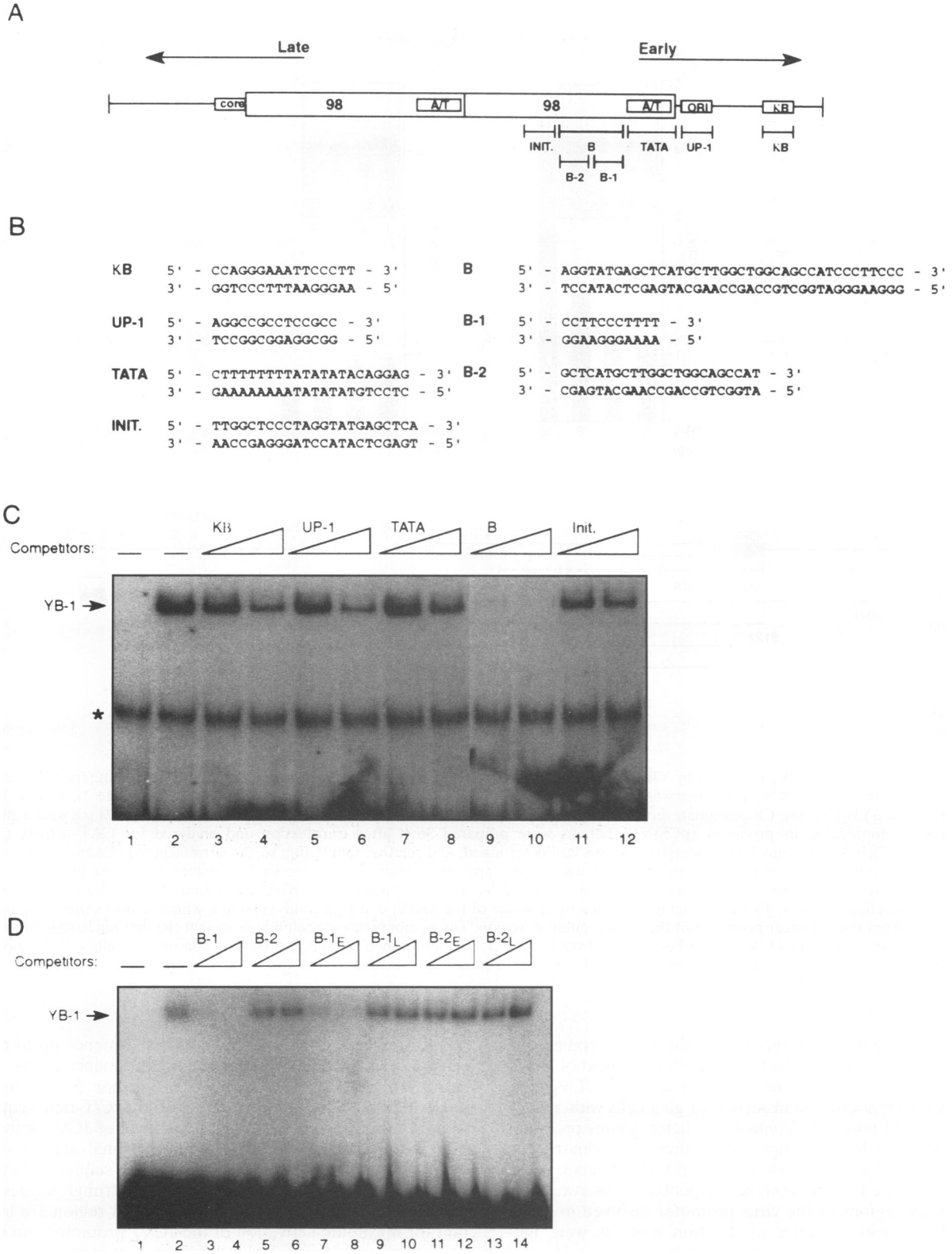

FIG. 2. Band shift analysis of bacterially produced B region-binding protein YB-1. (A) Structural organization of JCV enhancer/promoter and position of *cis*-acting regulatory motifs. (B) Sequence composition of the DNA fragments representing the *cis*-acting regulatory elements of JCV. (C) Band shift competition experiments examining binding of YB-1 to various regions of the JCV enhancer/promoter sequence. Binding reactions were carried out in a 30-μl reaction mixture in the presence of buffer containing 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.9]), 4 mM Tris (pH 7.5), 60 mM KCl, 5 mM $MgCl_2$, 0.8 mM dithiothreitol, and 0.5 μg of poly(dI-dC). DNA protein complexes were allowed to form during a 30-min incubation on ice as described previously (22) in the presence of 2 μg of bacterially produced proteins by using double-stranded B oligonucleotide as probe. Competitions were performed with 25 and 50 ng of the indicated competitor oligonucleotides. The arrow indicates the position of the specific DNA-protein complex, and the asterisk indicates the nonspecific and irreproducible band. (D) Band shift competition experiment to evaluate binding of YB-1 to the single- and double-stranded oligonucleotides derived from nonoverlapping regions of the B domain. E and L refer to the early and late strands of each respective oligonucleotide.

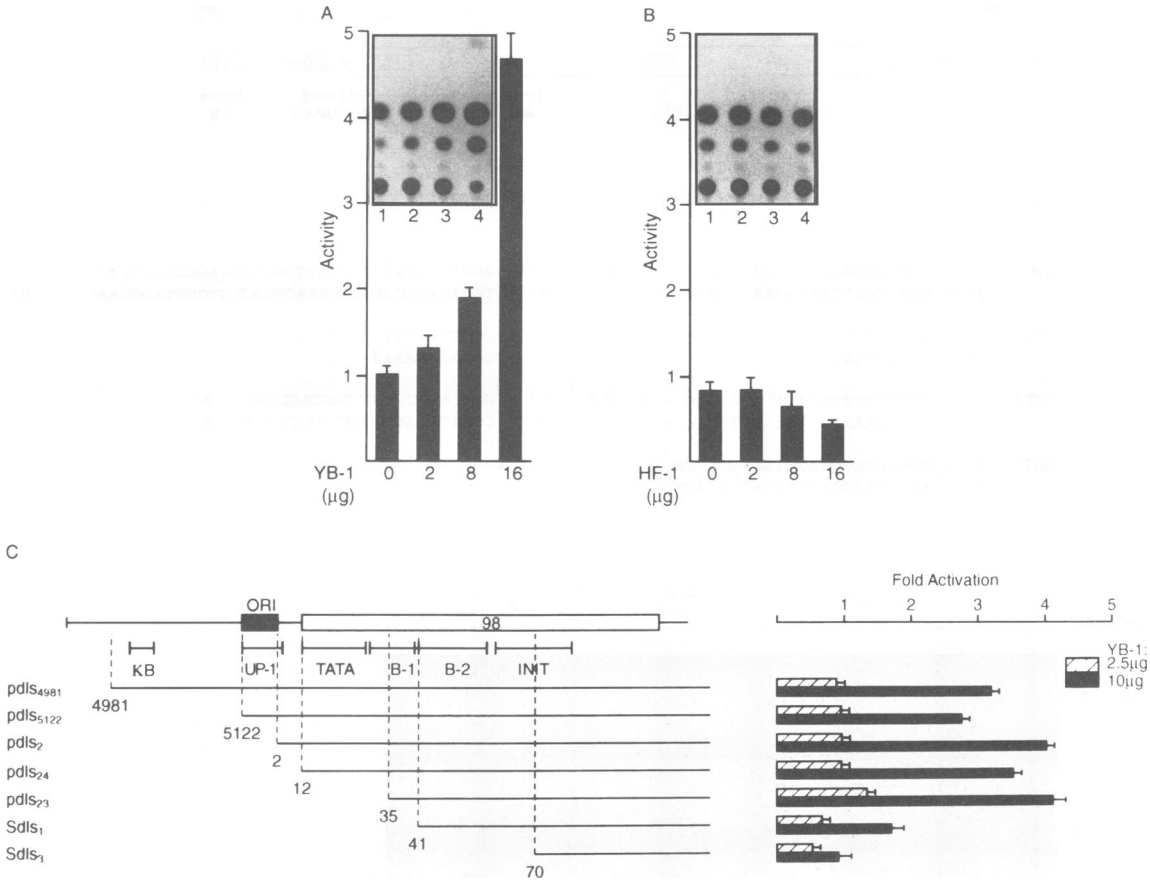

FIG. 3. Activation of the JCV_L promoter by YB-1 in glial cells. (A) Three micrograms of the plasmid $pJCV_L$-CAT containing the CAT reporter gene under the control of the JCV_L promoter was introduced into U-87MG glioblastoma cells alone (lane 1) or with 2 (lane 2), 8 (lane 3), and 16 (lane 4) μg of pYB-1 by the Ca phosphate precipitation method (16). The final DNA concentration was adjusted to 20 μg with a plasmid containing the cytomegalovirus promoter (pCMV). Extracts were prepared 36 h after transfection and analyzed for CAT activity (inset). Conversion of [^{14}C]chloramphenicol to its acetylated forms was determined, and relative stimulation by the cotransfected plasmids is shown. (B) Effect of HF-1 on transcription of the JCV_L promoter. A transfection protocol similar to that described for panel A utilizing pCMV-HF-1 was carried out. (C) Effect of 5′ deletion mutations on induction of the JCV_L promoter by YB-1. (Left) Organization of the JCV_L promoter region in $pdls_{4981}$ and deletion mutants. This construct was created by cleavage of the $pJCV_L$-CAT plasmid with *Sac*I, which removes the 98-bp internal DNA fragment from the enhancer/promoter of the virus regulatory region. The numbers below each line represent the first nucleotide of the JCV promoter present in each clone (14). (Right) Fold transactivation by YB-1 as determined by transient transfection of glial cells with 3 μg of each mutant promoter in the presence of 2.5 and 10 μg of pYB-1 expressor plasmid.

glial cells. Furthermore, it seems that the region comprising the cold shock domain and the first acidic/basic repeat of YB-1, contained in HF-1, is insufficient to exert its activity on JCV_L gene transcription. Transient cotransfection of glial cells with a CAT reporter plasmid under the control of a heterologous promoter derived from the herpes simplex virus thymidine kinase gene (HSV-tk) revealed that YB-1 exerts no significant alteration in the basal activity of the HSV-tk promoter (unpublished observations).

To identify regions of the virus promoter involved in mediating YB-1 activity, a series of deletion mutants were employed. These mutants were constructed in a reporter plasmid containing only one copy of the 98-bp repeat ($pdls_{4981}$) (Fig. 3C). Cotransfection of each of these constructs with the YB-1 expression plasmid into the glial cells allowed the determination of the importance of the B region in activation of the JCV_L promoter by YB-1. Deletion of 5′ JCV_L promoter sequence from nucleotide 4981 to 5122, which removes a 141-nucleotide sequence containing the NF-κB binding site, had no effect on the extent of JCV_L promoter activation by YB-1. Similarly, further removal of the sequence up to nucleotide 35 (dls_{23}) exhibited no substantial influence on the YB-1 activation of the JCV_L promoter. Further 5′ deletion to nucleotide 41 (dls_1), which removes the C/T-rich sequence found in the B-1 region, reduced the level of JCV_L activation by YB-1. Further decrease in transcriptional activity of the JCV_L was obtained upon removal of the sequence between nucleotides 41 and 70 (dls_3). These results strongly suggest that the nucleotide sequences located in the B-1 region are important for maximum activation of the JCV_L promoter and that a secondary target that is located downstream from B-1 may also respond to YB-1 activation.

Additional experiments performed with the B region cloned into a heterologous promoter showed no responsiveness to YB-1, even at high concentrations (data not shown). These data suggest that YB-1 may require a more native promoter context for transcriptional activation, in terms either of position or relationship to either *cis* sequences or their participant *trans*-acting factors.

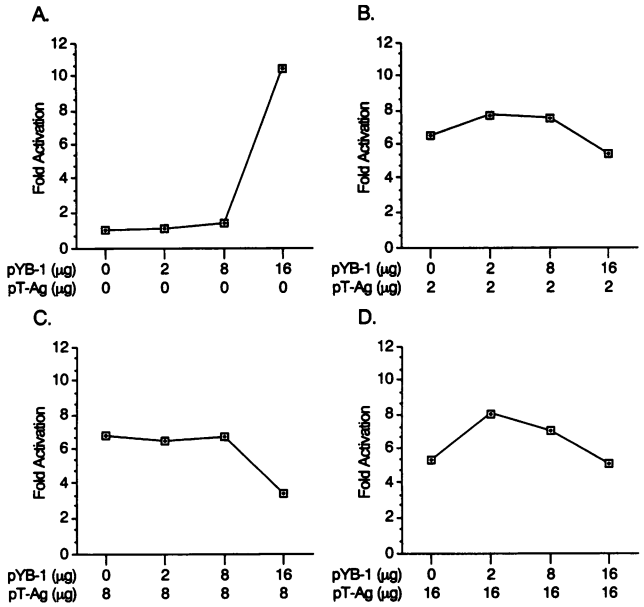

FIG. 4. Effect of JCV T antigen and YB-1 on the JCV_L promoter activity in U-87MG glial cells. Three micrograms of reporter plasmid ($pJCV_L$-CAT) was transfected alone or cotransfected with 2, 8, and 16 μg of pYB-1 in the absence (A) or presence of 2 (B), 8 (C), and 16 (D) μg of T antigen expressor plasmid pJC-T. The final amount of DNA was brought to 35 μg with pCMV plasmid, ensuring equal promoter levels in all samples. Extracts were prepared 48 h posttransfection and analyzed for CAT enzyme activity.

Effect of YB-1 and T antigen on JCV_L promoter activity. In previous studies, we demonstrated that expression of the JCV_L promoter is increased by the virus early protein T antigen in glial and nonglial cells (25). The ability of JCV T antigen to activate late gene transcription is independent of an increase in DNA replication. To evaluate the possible effect of YB-1 on T antigen-induced transcription of JCV_L promoter, we employed double and triple transfections of glial cells with constant amounts of JCV_L reporter construct and various amounts of YB-1 and T antigen expressor plasmids.

In the absence of virus T antigen, high levels of CAT activity were detected only when cells received 16 μg of YB-1-producing plasmid (Fig. 4A). In the absence of YB-1, addition of T antigen expressor plasmid at different concentrations increased the level of JCV_L promoter activity, reflecting transactivation of the JCV_L promoter by T antigen (Fig. 4, first datum point in panels A to D). Addition of YB-1 expression plasmid at suboptimal concentrations (2 and 8 μg) to the transfection mixture containing T antigen had no significant effect on the extent of the JCV_L promoter activities as mediated by 2 or 8 μg of pT-Ag (Fig. 4, compare the second and third datum points with the first datum point in panels B and C). Under similar conditions, at higher concentrations (16 μg), YB-1 diminished the T antigen-induced levels of JCV_L transcription, suggesting that these two proteins may function in a mutually exclusive manner (Fig. 4, compare the fourth datum point with earlier points in panels A to D). Interestingly, at suboptimal concentrations, pYB-1 was able to improve transcription of the JCV_L promoter as mediated by 16 μg of pT-Ag (panel D, first and second datum points).

These data are consistent with a model that YB-1, by interacting with T antigen, dilutes the excess amount of T antigen in the transfected cells, thus modulating the effect of T antigen on the virus promoter. In support of this concept, recent in vitro DNA binding studies in our laboratory indicated that T antigen modulates interaction of YB-1 with DNA and that the carboxyl terminus of YB-1 is important for T antigen to exert this activity. These observations suggest that the interaction of YB-1 and T antigen may play a role in regulating JCV gene expression. More recently, studies similar to those described above were carried out with the deletion mutant dls_1, and the results indicated that the YB-1 binding site within the B-1 region is important for the interplay between YB-1 and T antigen (7a). Currently, experiments are in progress to evaluate the biological significance of these findings and the role of YB-1 and T antigen in virus gene expression of the early and late phases during the JCV lytic cycle. Initial observations suggest that YB-1 may exert a negative effect on virus early RNA synthesis (7b).

Expression of YB-1 in glial and nonglial cells. To investigate cell type diversity of YB-1 gene expression, Northern (RNA) blot analysis of RNAs derived from various cell types was performed. To eliminate cross-hybridization with other members of the YB-1 family, probing and washing were performed under very-high-stringency conditions. As shown in Fig. 5A (right panel), a major 2.0-kb RNA species was detected in every cell used in this study. From the intensity of the 2.0-kb band, it appears that, with the exception of HL60 and Daudi cells, which showed a reduced level of YB-1 RNA, and BJAB cells containing an elevated level of this transcript, YB-1 mRNA was present at approximately similar levels in every cell line tested. Prolonged exposure of the blot revealed a minor 4.5-kb RNA species with a similar intensity in the cells (data not shown). Ethidium bromide staining showed that equal amounts of RNA were loaded on the gel (Fig. 5A, left panel).

To assess the developmental expression of YB-1 in neural tissue, RNAs from mouse brain were prepared and hybridized to YB-1 cDNA by the Northern blot technique. The results of this study (Fig. 5B) revealed the expression of a major 2-kb species that was more abundant in 3- and 7-day postnatal brain tissues (right panel, lanes 1 and 2). The level of YB-1 RNA was substantially decreased in RNA derived from 18-, 30-, and 60-day postnatal brain tissues (right panel, lanes 3 to 5). Longer exposure of the autoradiogram also revealed the presence of a 4.5-kb RNA species in mouse brain tissue at all ages (data not shown). Figure 5B (left panel) illustrates ethidium bromide staining of the RNA on nitrocellulose filters used in Northern hybridization. It should be mentioned that results from Northern blot analysis of RNA derived from human brain cells at two different stages of development have also shown the accumulation of the 2.0- and 4.5-kb transcripts, although the intensity of the 2.0-kb YB-1 RNA was lower in young adult tissues than that obtained in infant tissues (18a). To measure the distribution of YB-1 transcript in tissue, total RNAs isolated from a panel of mouse tissues were used in Northern blot analysis. As shown in Fig. 5B (right panel, lanes 7 to 11), with the exception of liver, nonneural tissues expressed high levels of YB-1 mRNAs.

These observations indicate that a major 2.0-kb YB-1 RNA species is produced in neural and nonneural cells and tissues and that expression of the 2.0-kb transcript is down-regulated during brain development.

JCV infection occurs during childhood in the majority of the population, but only during immunosuppression does this virus become pathogenic and cause progressive multifocal leukoencephalopathy. This virus replicates exclusively in glial cells, the myelin-producing cells of the central nervous system. The mechanisms that determine glial cell-specific expression of virus genes are not entirely understood. It is postulated,

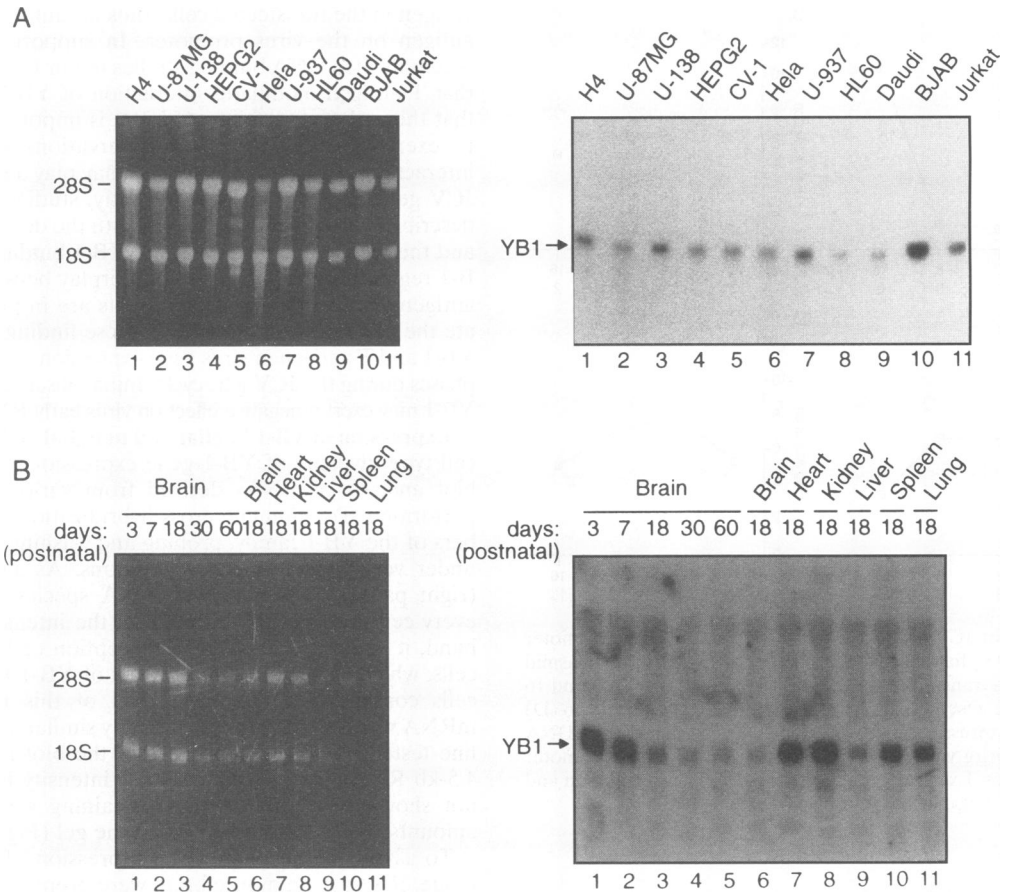

FIG. 5. Northern analysis of YB-1 RNA. Total RNA was extracted from various cell lines and from brain tissue at various stages of development by the modified guanidine isothiocyanate method (8). Approximately 20 μg of total RNAs isolated from various human cell lines (A, right); mouse brain tissue at 7, 14, 18, and 30 days after birth; or various tissues from an 18-day-old mouse (B, right) were analyzed on denaturing formaldehyde agarose gels (3) and hybridized to the YB-1 cDNA probe after transfer to nitrocellulose.

however, that a regulatory pathway that includes participation of glial and nonglial DNA-binding transcription factors modulates the host-specific replication of JCV (5, 38).

The data presented here have led to the identification of a DNA-binding protein, called YB-1, that binds specifically to the JCV DNA sequence and has the capacity to up-regulate transcription of the virus late promoter in glial cells. Results from the cotransfections with plasmids producing YB-1 and T antigen demonstrated that, at optimum levels, expression of T antigen in glial cells decreases the extent of transactivation mediated by YB-1. Studies are currently under way to determine whether or not direct competition between these proteins for association with DNA may be responsible for the reduction of YB-1-mediated transactivation by T antigen. It is likely that the reduction of virus transcription is achieved upon direct interaction between YB-1 and T antigen. Our recent in vitro binding studies have revealed that communication between YB-1 and T antigen may determine the DNA binding activity of YB-1 (7b). Experiments are in progress to assess the importance of YB-1–T antigen interaction in replication of JCV in lytically infected glial cells.

Previously, we demonstrated that, similar to the early promoter, basal activity of the late promoter is restricted to glial cells (25). With the notion that YB-1 is ubiquitously expressed in every cell and tissue, one might anticipate the involvement of a negative regulatory mechanism in nonglial cells that interferes with the positive activity of YB-1 on the basal and T antigen-induced levels of virus late gene expression. Whether or not YB-1 plays a role in the latency and reactivation of JCV in neural and nonneural tissues or participates in establishing latent JCV infection in these tissues remains to be investigated. It should be mentioned that the brain, kidney, and lymphocytes (B cells) are assumed to be the primary sites of JCV infection and latency (26, 27). Thus, it is intriguing to speculate that under immunocompromised conditions, reactivation of JCV gene expression and replication may be achieved, at least in part, by active participation of regulatory factors that stimulate the virus lytic cycle. Experiments are in progress to investigate the regulation of YB-1 in normal and immunocompromised animals and to examine its possible role in transcription of JCV promoters in neural and nonneural tissues.

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