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Nuclear factors in human brain cells bind specifically to the JCV regulatory region

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The human polyomavirus, JCV, differs from other papovaviruses in its tissue tropism for human glial cells. Transcription of the early region of the virus, at least in part, contributes to the tissue specificity of JCV. In this study, we have synthesized oligonucleotides which span the JCV 98 bp repeat unit. Using gel mobility shift and UV cross-linking assays, we have demonstrated that four proteins from a human fetal brain extract interact specifically with the JCV promoter/enhancer. Two proteins of 82 kd and 78/80 kd recognize the 5'- and 3'-terminal regions of the JCV 98 bp repeat sequence, respectively. The mol. wt of these proteins are similar in HeLa and brain extracts. In contrast, the proteins which recognize the central region of the 98 bp enhancer are distinct in HeLa (85 kd) and fetal brain (45 kd) extracts. The possible role of these proteins in tissue-specific expression of the JCV early promoter in brain cells is discussed.

Key words: DNA-binding proteins/JC virus/brain nuclear extract/UV cross-linking

Introduction

Many eukaryotic genes have been described which are regulated at the level of transcription and expressed only in certain cells and tissues (Gillies *et al.*, 1983; Grosschedl and Baltimore, 1985). A number of studies have suggested that DNA enhancer sequences are responsible for tissue-specific gene expression (Laimins *et al.*, 1982; Scholer and Gruss, 1984; Gillies *et al.*, 1984; Picard and Schaffner, 1984; Mercola *et al.*, 1985; Ott *et al.*, 1986; Ohlsson and Edlund, 1986). In support of this argument, it has been demonstrated that the immunoglobulin heavy chain gene (Faulkner *et al.*, 1986; Singh *et al.*, 1986; Wasylyk and Wasylyk, 1986) and the polyoma and SV40 enhancers contain multifunctional domains, each interacting with particular tissue-specific cellular factors (Kovesdi *et al.*, 1987; Wildemen *et al.*, 1986; Davidson *et al.*, 1986). Presumably, the level of RNA synthesis in different cell types is governed by the relative abundance of tissue-specific transcription factors which interact with *cis*-acting DNA sequences (Maniatis *et al.*, 1987; McKnight and Tjian, 1986).

It has been estimated that 25 000 different genes are turned on in the mammalian nervous system, most of which are neural specific and coordinately regulated during brain development (Milner and Sutcliff, 1983). In order to increase our understanding of neural gene regulation, we have chosen the neurotropic virus, JCV, as a model system. JC is a human polyoma virus which replicates exclusively in human fetal glial cells. This virus is often associated with progressive multifocal leukoencephalopathy and is suspected to be the causative agent of the disease (Padgett and Walker, 1973; Padgett *et al.*, 1977a,b).

The genomic organization of JCV is very similar to that of SV40 as determined by their DNA sequence homology (Frisque *et al.*, 1984). The structure of the control region of JC (m.p. 5130-271), in contrast, differs in sequence from the other papovaviruses. The control region contains two 98 bp repeats, shown by transient expression assay to be responsible for its unique tissue tropism (Kenny *et al.*, 1984; Feigenbaum *et al.*, 1987). In this report, we have analyzed protein factors present in human fetal brain nuclear extracts which bind specifically to the JCV enhancer/promoter region. For comparison, the widely used HeLa nuclear extract was included in these analyses. At least four proteins present in brain extracts specifically bind to the JCV enhancer/promoter region. Of particular interest is the observation that proteins which recognize the central region of the 98 bp repeat are distinct in HeLa (85 kd) and fetal brain (45 kd) extracts.

Results

Localization of DNA-protein interactions within 98 bp enhancer/promoter regions

To identify cellular proteins which interact with the JCV enhancer sequence, we first performed gel electrophoresis of DNA-protein complexes. This method is remarkably sensitive and can detect specific DNA-binding proteins with affinity constants of 10^5/M (Fried and Crothers, 1981). To minimize the binding of non-specific proteins to the DNA probe, poly dI-dC was added to the binding reaction (Singh *et al.*, 1986). Figure 1 illustrates the organization of the control region of JCV and the DNA fragments within the 98 bp repeat region which were used to assay for binding of nuclear factor(s). These DNA fragments are designated AB (m.p. 196-228), CD (m.p. 181-207), EF (m.p. 157-180), GH (m.p. 133-160) and IJ (m.p. 110-133). The mobilities of these fragments alone or in complex with proteins derived from HeLa or brain extract is shown in Figure 2. In the presence of the extracts, migration of DNA fragments was retarded, resulting in the appearance of slower migrating bands when HeLa extract was used as a source of DNA-binding proteins (Figure 2, lane 2). At least one DNA-protein complex was formed with fragments AB, CD and EF (Figure 2, panels A-C). In human brain extracts AB, CD and EF gel-shift complexes migrated with slightly faster mobility and were significantly less intense than complexes formed in

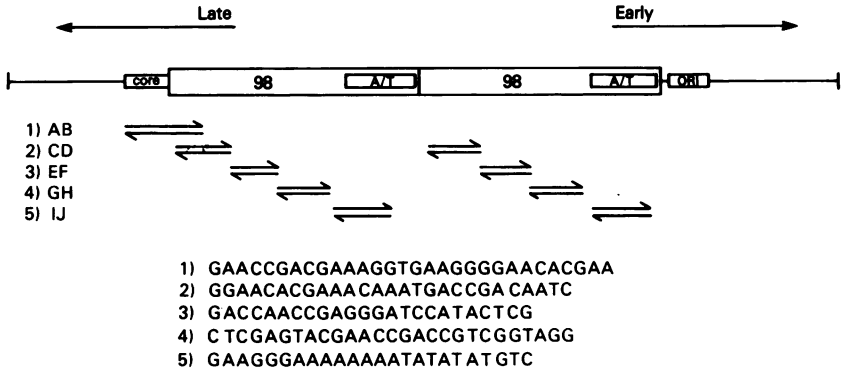

Fig. 1. JCV early promoter and DNA probe strategy. The top line displays an overview of the JCV control region including two 98 bp repeats, AT rich region, sequences analogous to SV40 'core' enhancer sequences and origin of DNA replication. The double-stranded oligonucleotide probes are indicated by short arrows: AB (m.p. 196–228), CD (m.p. 181–207), EF (m.p. 157–180), GH (m.p. 133–160) and IJ (m.p. 110–133) beneath, the nucleotide sequence of each oligonucleotide is indicated.

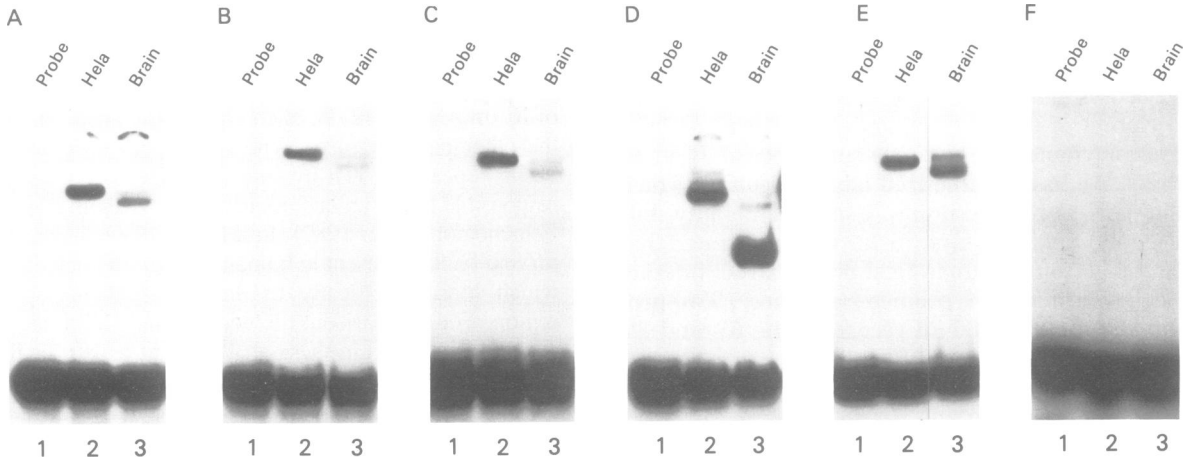

Fig. 2. Gel-shift analysis of the JCV enhancer/promoter oligonucleotides *in vitro* in human fetal glial and HeLa extracts. The end-labeled double-stranded oligonucleotide fragments were incubated in nuclear extracts from HeLa and brain cells and the resulting complexes were separated from the unbound input fragment by electrophoresis on native polyacrylamide gels as described in Materials and methods. The five oligonucleotide probes (AB, CD, EF, GH and IJ) are shown in **panels A–E**, respectively. The oligonucleotide used in **panel F** comes from the JCV coding region and serves as a negative control. **Lane 1**, no extract added; **lane 2**, nuclear extract from HeLa cells; **lane 3**, nuclear extract from human primary fetal glial cells.

HeLa extracts (compare Figure 2, lanes 2 and 3, panels A–C). Competition experiments using these three fragments indicated that they may interact with similar proteins in both extracts (data not shown). When the GH DNA fragment was incubated with the HeLa extract one major gel-shift band was observed (Figure 2, panel D, lane 2). In human brain extracts, a gel-shift band of equal intensity but altered mobility was detected with the GH probe (Figure 2, panel D, lane 3). Fragment IJ, spanning the AT-rich region of the JCV enhancer protein, produced complexes in HeLa and brain extracts with similar intensity and gel-shift mobility (Figure 2, panel E, lanes 2 and 3). As a negative control, a double-stranded oligonucleotide homologous to the JCV early coding region (m.p. 4847–4895) was tested. No binding activity, as assayed by the gel-retardation assay, was detected with either the HeLa or fetal brain extracts.

Specificity of DNA–protein complex formation

The above experiments demonstrate that the JCV enhancer/promoter is composed of multiple protein binding domains, each of which can bind to nuclear protein(s). In view of the great dissimilarity in the mobilities of the GH complexes formed in HeLa and in brain extracts, we focused

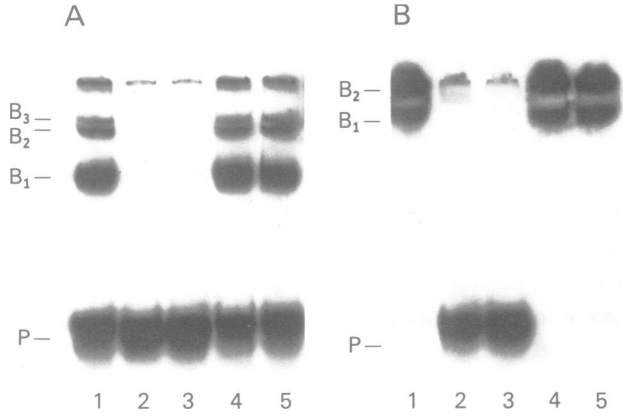

Fig. 3. Competition analysis of GH binding factors with JCV enhancer domains. Binding reactions were carried out in a 30 μl final volume with 20 μg of nuclear extract derived from primary human fetal glial cells (**panel A**), or HeLa cells (**panel B**), with 10–20 ng of end-labeled GH probe 20 000 (50 000 c.p.m.) and 15 μg poly dI-dC. **Lane 1**, no competitor DNA added; **lanes 2** and **3**, binding reactions in the presence of 10- and 100-fold excess of unlabeled GH fragment, respectively; **lanes 4** and **5**, binding reactions in the presence of 100-fold excess of EF and IJ fragments, respectively. P demonstrates the position of free probe and B1–B3 indicates the migration of different DNA–protein complexes.

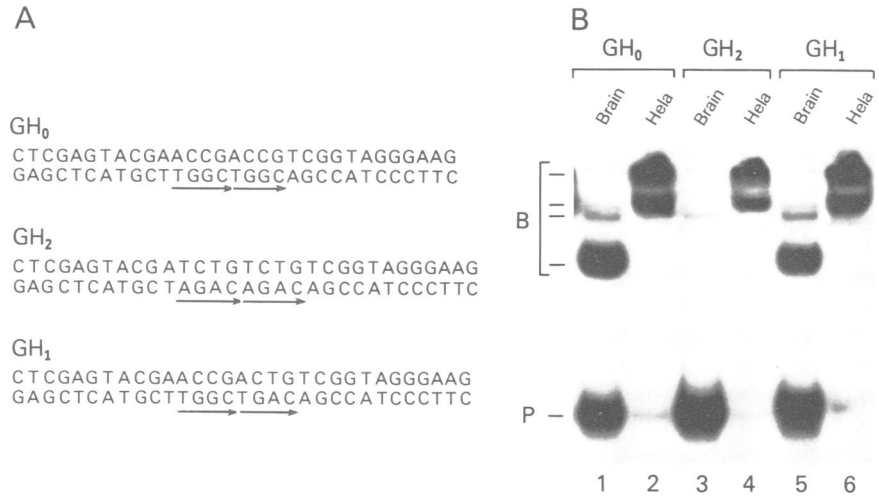

Fig. 4. Localization of the GH binding domain at the nucleotide level. Binding reactions were performed as described in Materials and methods. 20 μg of either HeLa or fetal brain extract were mixed with 15 μg of poly dI-dC and 20 ng of end-labeled DNA probe. (**A**) The nucleotide sequence of the three double-stranded oligonucleotides is shown. The position of the internal repeat and its corresponding mutants are indicated by arrows. (**B**) Gel-shift analysis of each of the fragments in HeLa (**lanes 2, 4** and **6**) or human brain (**lanes 1, 3** and **5**) extracts. P and B indicate the position of the free and bound DNAs respectively.

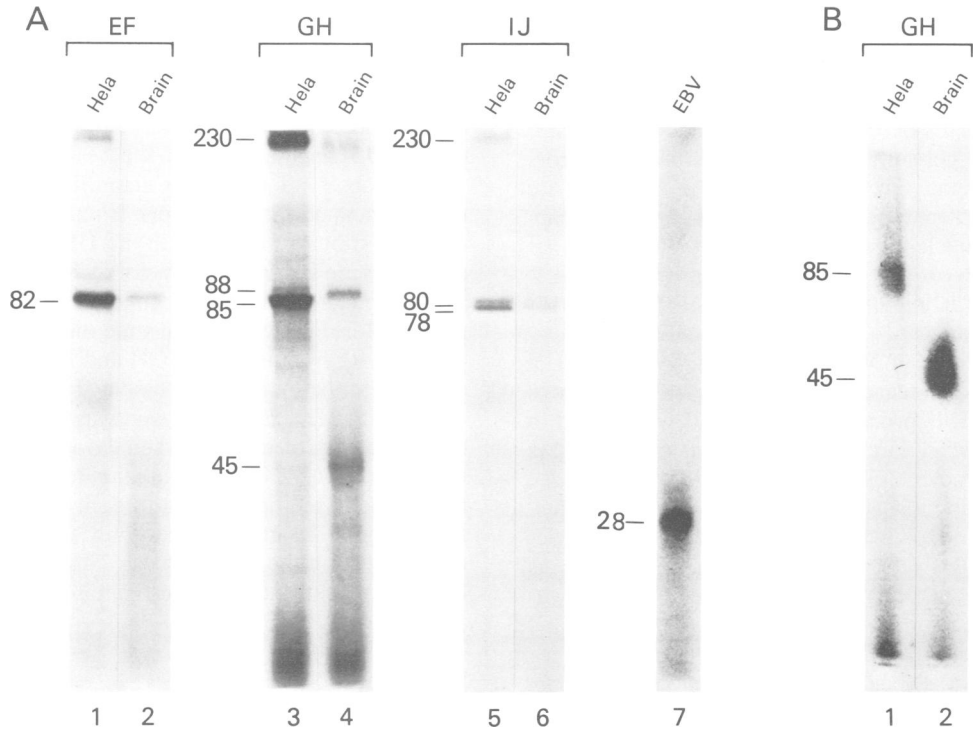

Fig. 5. Identification of JCV enhancer binding proteins by UV cross-linking. Binding of BUdR incorporated oligonucleotides to the proteins and UV cross-linking was performed according to the procedures described in Materials and methods. (**A**) Binding of HeLa factors (**lanes 1, 3** and **5**) or brain factors (**lanes 2, 4** and **6**) to the three different domains of the JCV enhancer seuqences, EF, GH and IJ (see Figure 1) respectively. **Lane 7**, binding of purified EBNA protein to its corresponding oligonucleotide binding site. (**B**) Analysis of proteins associated specifically with the major GH gel-shift complexes formed in HeLa (**lane 1**) or brain (**lane 2**) extracts (see Figure 2, panel D). Gel-shift bands representing DNA–protein complexes were excised from the native gel. Proteins were then eluted and analyzed in a 12% SDS–polyacrylamide gel.

on this region for further analysis. To confirm the specificity of protein(s) binding to the GH sequence, competition experiments using unlabelled EF or IJ fragments were performed (Figure 3). Addition of a 10- or 100-fold excess of the unlabelled GH fragment completely abolished the association of the GH probe DNA with the HeLa or brain proteins (Figure 3, panels A and B, compare lanes 1 with 2 and 3). In contrast, incubation with a 100-fold excess concentration of two fragments representing regions 5′ or 3′ to the GH sequence (fragments EF and IJ) had no significant effect on the complex formation with the GH probe (Figure 3, panels A and B, lanes 4 and 5). These results suggest that the factors in HeLa and brain extracts which recognize the GH region of the enhancer have sequence-specific DNA binding properties and do not possess strong affinity for sequences flanking the GH region.

Factors in brain extracts recognize internal tetranucleotide repeats

To distinguish the GH binding factors in brain from their HeLa counterparts, we attempted to further localize the sites of interaction. We utilized two mutant DNA fragments, similar to GH, in the binding assay. We focused on the central nucleotides of the GH fragment where there exists a repeat of the tetranucleotide $\frac{ACCG}{TGGC}$ (Figure 4, panel A). The nucleotide sequence of the two mutants are shown in Figure 4, panel A. Mutant GH_1 contains a G→A transition in the downstream TGGC repeat (GH_1). Mutant GH_2 contains one transversion (T→A) and one transition (G→A) in both repeats. These fragments, together with the wild-type fragment (GH_0) were labelled and separately mixed with HeLa or brain extracts. The results of this experiment are illustrated in Figure 4, panel B. Single point mutations in the tetranucleotide repeat (GH_1), did not affect the binding of HeLa or brain factors. With mutant GH_2 fragment, however, we observed a significant reduction in the binding of the brain-specific factor (lanes 1, 3 and 5) but not the HeLa factor (lanes 2, 4 and 6). In parallel binding experiments were conducted under conditions where only 50% of the probe was bound. Again no significant decrease in binding activity of the mutants was detected when HeLa extract was used as a source of proteins (data not shown). This result suggests that GH-specific binding proteins in HeLa cells differ from their counterparts in brain cells with regard to their target DNA sequence. This observation, consistent with the size difference of the gel-shift bands, implies that the factors which recognize the GH domain of JCV enhancer are distinct. At the present time we have not yet accurately mapped the binding nucleotides of the HeLa factor.

Identification of JCV enhancer binding protein by UV cross-linking analysis

For direct analysis of JCV enhancer binding factors, UV cross-linking of the proteins with the specific target sequences was performed. This procedure has recently been used to identify proteins which bind to specific regulatory sequences of eukaryotic promoters (Chodosh *et al.*, 1985; Jeang *et al.*, in preparation). In this analysis, short DNA fragments, uniformly incorporated with [α-^{32}P]dCTP and bromodeoxyuridine (BUdR) were mixed with nuclear extracts as described in Materials and methods. The entire reaction was then UV-irradiated for 2−3 min, treated wtih DNase and analyzed by gel electrophoresis.

To demonstrate that a short oligodeoxynucleotide does not significantly alter the electrophoretic mobility of the protein to which it is associated, a 30 bp fragment spanning the origin of Epstein−Barr virus (EBV) (ori-P) was mixed with HeLa extract and 50 ng of the purified EBNA protein (kindly provided by Dr T.Jeang). Results in Figure 6, lane 7 showed that no significant difference was observed upon association of the 30 bp fragment with the small 25 kd protein. Furthermore, incorporation of BUdR into the labelled probe did not change the binding characteristics of the DNA to the specific probe (see also Chodosh *et al.*, 1986). Therefore, the authentic size of the DNA-bound protein can be estimated by electrophoretic mobility.

To determine the sizes of the proteins interacting with domains of the JCV enhancer, labelled BUdR containing fragments EF, GH and IJ were separately incubated with brain or HeLa extract. After UV irradiation, each sample was digested with DNase and resolved by SDS−PAGE. With the EF fragment, a single major band of 82 kd was observed in both the HeLa and brain extracts (Figure 5, lanes 1 and 2). From the intensity of the band it appears that this protein is present at higher concentrations (3- to 5-fold) in HeLa extract than in brain extract. A similar size protein was also detected when CD fragment was used as a probe (data not shown). The oligonucleotide probe IJ, spanning the A/T-rich region interacts with at least two major proteins of 78 and 80 kd in both extracts. These proteins also appear to be less abundant (5- to 10-fold) in the brain extract, as determined by the relative intensities of the bands (Figure 5, lanes 5 and 6). In addition to the 70/80 kd doublet, a weaker band of 230 kd was also detected in the HeLa extract. The central domain of the JCV enhancer corresponding to fragment GH bound at least two proteins in HeLa extract, with mol. wts of 85 and 230 kd (Figure 5, lane 3). In brain extracts, two major bands of 45 and 88 kd were reproducibly observed (Figure 5, lane 4). To demonstrate that the major GH-binding proteins in HeLa (85 kd) and in brain (45 kd) are responsible for the altered mobility of the GH fragment in the gel-shift assay (Figure 2, panel D), the labelled BUdR incorporated GH fragment was mixed with HeLa or brain extracts. After cross-linking, the nucleoprotein complexes were resolved on a native acrylamide gel, the gel-shift bands excised and analyzed on a 12% SDS gel. The proteins extracted from the HeLa and brain complex migrate as 85 kd and 45 kd polypeptides, respectively (Figure 5, panel B).

These observations directly demonstrate the interaction of two distinct nuclear factors, one from HeLa (85 kd) and the other from brain (45 kd) with the GH core JCV enhancer sequences. In contrast, the factors which recognize the EF and IJ regions appear to be identical. In addition, the results presented in Figure 5 demonstrate the binding of a 230 kd protein with fragments EF, GH and IJ when HeLa extract was used as the source of the binding factors (Figure 5, lanes 1, 3 and 5). The 230 kd protein was also detected following incubation of the GH fragment with the brain extract (Figure 5, lane 4). Thus, in addition to the smaller size proteins which specifically bind to each region, a single high mol. wt protein may interact with several regulatory sequences within the 98 bp repeat. The nature of these proteins is currently under investigation.

Discussion

Results presented here demonstrate that multiple DNA-binding regions within the JCV 98 bp enhancer/promoter ('A', 'B' and 'C') intract with cellular proteins. Region 'B', represented by oligonucleotide probe GH, is located at the central portion of the 98 bp repeat and interacts with proteins which are found specifically in brain cells. In contrast, DNA sequences corresponding to region 'A' (fragment AB, CD and EF) and region 'C' (fragment IJ) appear to bind similar size proteins in both HeLa and brain extracts, as determined by mobility on SDS−polyacrylamide gels.

The 'A' region contains two CAAT box-like sequences (CAAT and CAAAT). We have tested its interaction with the CTF transcription factor. This protein factor has recently been purified and shown to stimulate transcription of several class II promoters containing CAAT-like sequences (Jones

et al., 1987). Interestingly, CTF also stimulates replication of adenovirus DNA replication *in vitro* and appears to be identical to the previously identified replication factor, NF-1 (Rosenfeld and Kelly, 1986). Using the DNA mobility shift assay, we were unable to detect any association between region 'A' of the JVC 98 bp repeat containing the CAAT-like sequences (fragment CD) and purified CTF (a gift from Dr T.Kelly) (our unpublished data). Consistent with these findings, the protein which interacts with the JCV sequences has an apparent mol. wt of 82 kd which is different from the size of CTF/NF-1 protein (Rosenfeld and Kelly, 1986).

The proteins which recognize region 'B', the central portion of the JCV 98 bp repeat (fragment GH), appear to exhibit a tissue-dependent diversity. While DNase I protection maps obtained using a DNA fragment spanning the B-domain are similar in both HeLa and brain extracts (data not shown), the protein factors recognizing this region are distinct as determined by SDS−PAGE (Figure 5B). In brain extracts this region interacts with protein components of 45 and 88 kd, as determined by UV cross-linking experiments. The corresponding proteins identified in HeLa extract which interact with this region are 85 and 230 kd. Results of gel-shift experiments using oligonucleotides containing base substitutions within the tetranucleotide repeats $\frac{ACCG}{TGGC}$ suggest this sequence is important for the interaction of the 45 kd component in brain extracts. Base substitution within the tetranucleotide repeats did not have a significant effect on the binding of the HeLa 85 and 230 kd proteins suggesting that the recognition sequence of these proteins might be distinct.

The insertion of the GH fragment (region 'B') upstream of the SV40 21 bp repeats restored transcriptional activity of the enhancerless SV40 promoter in HeLa cells (*in vivo*) and in HeLa extracts (*in vitro*) (Khalili *et al.*, in preparation). These results suggest that the GH domain of the JCV enhancer is a functional transcriptional regulatory sequence. Analogous studies using human fetal glial cells and human fetal brain extracts are currently under investigation.

The third region of the 98 bp repeat, region 'C', is recognized specifically by a 78 kd protein present in HeLa and in brain extracts. This region is rich in adenosine and thymidine and may constitute a functional TATA box. Previous studies by Davidson *et al.* (1983) have demonstrated that a transcription factor specifically binds to the TATA box of the conalbumin and adenovirus major late promoter in the absence of RNA polymerase II. At present, it is not clear whether region 'C' of the JCV promoter interacts with the TATA binding factor(s) (Parker and Topol, 1984)

Experiments described here characterize the binding of specific protein factors for human brain tissue to the enhancer domain of JCV early promoter. Although the enhancer element of JCV is required for its tissue-specific activation in the cell, the results presented here do not demonstrate that these protein factors serve a functional role in promoter activation. More direct functional analyses are essential to characterize the roles of these protein factors in transcription and to identify specific protein−protein interactions these factors have with each other and/or RNA polymerase II. Studies are also in progress to purify the factors which recognize the three regions of the JCV 98 bp repeat and to assay their ability to activate transcription in an *in vitro* reconstituted system.

Materials and methods

Cells and cell extracts

Primary human fetal glial cells (PHFG) were prepared from 15−20 week old aborted fetuses by procedures previously described (Padgett, 1977). HeLa or PHFG cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (HeLa) or 3% (PHFG) fetal bovine serum. HeLa and PHFG nuclear extracts were prepared according to the method of Wildeman *et al.* (1985).

Preparation of radiolabelled DNAs

The oligodeoxynucleotide probes used in gel electrophoresis mobility shift assays were labelled with the Klenow fragment of DNA polymerase I and purified by PAGE. To obtain probe DNA for UV cross-linking studies, double-stranded oligonucleotides were labelled with [α-^{32}P]dCTP in the presence of BUdR using DNA polymerase I (Kornberg enzyme) according to standard methods (Maniatis *et al.*, 1982).

Gel electrophoresis DNA binding assay

Protein complexes were resolved on low ionic strength 9% polyacrylamide gels as described (Strauss and Varshavsky, 1984). Protein samples were incubated with 120−600 ng/ml (2000 c.p.m./ng) of end-labeled double-stranded oligonucleotides in the presence of 500 μg/ml poly dI-dC:poly dI-dC (Pharmacia) in a final volume of 30 μl. Incubations were carried out at room temperature for 30 min in 17% glycerol, 12 mM Hepes (pH 7.9), 50 mM NaCl, 5 mM $MgCl_2$, 4 mM Tris, pH 8.0 and 0.8 mM DTT. Samples were layered on top of low ionic strength 9% polyacrylamide (19:1) gels. Electrophoresis was carried out for 2−3 h at 180 V in 0.3 × TBE at 4°C. Gels were dried on Whatman 3MM paper and autoradiographed.

UV cross-linking

The oligodeoxynucleotides were ^{32}P-labeled by nick-translation in the presence of [^{32}P]dCTP, BUdR, dATP, dGTP and DNA polymerase I (Kornberg enzyme). Labeled oligonucleotides were separated from free nucleotides by gel filtrations and mixed with extracts under conditions described in the gel-shift assay. Reaction mixtures are first treated with UV (302 nm wavelength) for 2−3 min and then with DNase I (20 μg/ml) as described (Chodosh *et al.*, 1986). The molecular size of the complex was subsequently determined by SDS−PAGE.

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