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# Transcriptional Regulation of the Glial Cell-Specific JC Virus by p53

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The human polyomavirus JC virus is the etiologic agent of progressive multifocal leukoencephalopathy (PML). As the JC virus early promoter directs cell-specific expression of the viral replication factor large T antigen, transcriptional regulation constitutes a major mechanism of glial tropism in PML. It has been demonstrated that SV40 or JC virus large T antigen interacts with p53 protein and regulates many viral and cellular genes. In this study we found that p53 represses the JC virus early promoter in both glial and nonglial cells. To identify the cis-regulatory elements responsible for p53-mediated repression, deletional and site-directed mutational analyses were performed. Deletion of the enhancer region diminished p53-mediated transcriptional repression. However, point mutations of several transcription factor binding sites in the basal promoter region did not produce any significant changes. In support of this observation, when the enhancer was fused to a heterologous promoter, p53 reduced the promoter activity about three fold. These results indicate that the enhancer region is important for the repression of JC virus transcription by p53. Furthermore, coexpression of JC virus T antigen with a p53 protein abolished p53-mediated repression of the JC virus early promoter in non-glial cells, but not in glial cells. This finding suggests that T antigen interacts with p53 and regulates JC virus transcription in a cell-specific manner.

**Key words:** JC virus, Progressive multifocal leukoencephalopathy, p53, T antigen, Transcriptional repression, Enhancer

## INTRODUCTION

Progressive multifocal leukoencephalopathy (PML) is a fatal demyelinating disease that results from an oligodendrocyte infection caused by JC virus. JC virus selectively destroys oligodendrocyte, leading to multiple areas of demyelination and attendant loss of brain function (Astrom *et al.*, 1958; Zu Rhein *et al.*, 1965). Once a rare condition, PML is no longer infrequent, occurring in 5% of individuals with AIDS (Bacellar *et al.*, 1994). JC virus infection exists in a persistent state in kidney tissue and peripheral blood lymphocytes through the life of healthy individuals. In the setting of immunodeficiency, the virus infects and destroys oligodendrocytes, producing patches of myelin loss in subcortical white matter (Major *et al.*, 1992). Thus neuropathological features suggest that reactivated JC virus infection is specific for glial cells. Recent evidence suggests that JC virus is also associated with human tumors including astrocytomas (Webster and Major, 1997; Rencic *et al.*, 1996).

The JC virus early promoter directs cell-specific expression of the large T antigen, which is required for viral replication, and thus transcriptional regulation constitutes a major mechanism of the glial tropism of PML (Kenny *et al.*, 1984). The MH1 form of the JC virus promoter was directly isolated from the brain of a PML patient (Henson *et al.*, 1992), and its sequence found to diverge somewhat from that of the original Mad-1 promoter (Henson *et al.*, 1994). However, both MH1 and Mad-1 proximal promoters have sequence identity from the initiating codon for T antigen up to a pentanucleotide site just upstream of the viral TATA sequence.

The JC virus promoter is divided into an enhancer region important for promoter strength and a proximal promoter region which is able to direct glial cell-specific gene

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expression. We have previously shown that the proximal region contains two T antigen binding sites (LTa I and LTa II) and that T antigen produces glial cell-specific, divergent regulation of the JC virus basal promoter (Henson *et al.*, 1995; Kim *et al.*, 2000). The pentanucleotide and TATA sequences are important for this T antigen-mediated regulation. Since large T antigens from simian virus 40 (SV40) or JC virus have been demonstrated to form a physical complex with p53 (Dobbelstein and Roth, 1998; Jiang *et al.*, 1993), in the present study we investigated whether p53 regulates JC virus transcription and interacts with T antigen.

p53 is a 393 amino-acid transcription factor that regulates the normal cellular response to DNA damage (Kastan *et al.*, 1991). Its major known biochemical function is to activate the expression of genes whose promoters contain specific sequences that constitute p53 binding sites (El-Deiry *et al.*, 1992). It can also repress the expression of genes that lack binding sites (Mack *et al.*, 1993). The large T antigen of SV40 or JC virus inhibits p53-mediated activation of the p53-responsive promoter (Dobbelstein *et al*, 1998; Jiang *et al.*, 1993). In addition, p53 inhibits JC virus replication by preventing DNA unwinding by the large T antigen helicase (Staib *et al.*, 1996). However, the effect of p53 on JC virus transcription has not been clearly demonstrated up until now.

In the present study, we showed that p53 repressed the MH1 JC virus early promoter in both glial and non-glial cells. By site-directed mutagenesis and deletional analysis we confirmed that the enhancer region was important for the repression. Additionally, the enhancer sequences conferred p53-mediated repression on the heterologous promoter. T antigen de-repressed p53 activity in nonglial cells but did not affect p53-mediated repression in U87MG glioma cells, indicating differential interplay between p53 and T antigen in glial and non-glial cells.

## MATERIALS AND METHODS

### Cell culture and transient transfection assays

U87MG human glioma, HeLa human cervical carcinoma and Saos human osteosarcoma cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum(Hyclone), streptomycin, and penicillin. Transfection was performed by a standard calcium phosphate method. Cells ($2 \times 10^5$ in 60 mm-diameter dishes) were transfected with 4 μg of the reporter construct, 1 μg of pRSV β-gal, various amounts of the effector plasmid, and PUC19 plasmid to a total of 10 μg of DNA. For cotrans-fection with p53 or T antigen expression vector, one tenth moles of MH1long-luc were used. Plasmids used for transient transfection assays were prepared by using Qiagen (Santa Clarita, Calif., USA) columns. Cells were harvested after 48 h and luciferase or chloramphenicol acetyltransferase (CAT) assays were performed as pre-viously described (Kim *et al*, 1998; Kim *et al.*, 2000). To correct for differences in transfection efficiencies among different DNA precipitates, luciferase or CAT activity was normalized to β-galactosidase activity as determined by ONPG assay. Within T antigen expression experiments, luciferase values were normalized by protein concentrations as determined by a protein assay reagent from Bio-Rad, since T antigen regulates the promoters commonly used for internal controls (*e.g.* RSV promoter). All transfection assays were performed at least three times in duplicate.

### Plasmids

The pMH1long-luc reporter construct contains the 408-bp upstream sequence of the JC virus large T antigen gene fused to the firefly luciferase gene. Base substitutions or deletions in the promoter region of the JC virus were generated in the context of the 408 bp upstream sequence, using a QuickChange PCR-based site-directed mutagenesis kit (Stratagene, USA) according to the manufacturer's guidelines. The oligonucleotide sequences used for generating mutants of pentanucleotide, TATA and Sp1 sites are as described previously (Henson *et al.*, 1995; Kim *et al.*, 2000). To generate the deletion mutants of the enhancer sequences, the following oligonucleotides were used: 5' GCTTTTTGCAAAAAATTACTGC 3' for sense strand primer and 5' GCTTTCCACTTCCCCTTGCTCCC TACCTTCCCTTT 3' for antisense strand primer. In addition, enhancer sequences were synthesized by PCR from MH1long-luc using primers 5' CCCAAGCTTTGCTTTGT TTACTTGTGTC 3' for sense strand primer and 5' ACATG CATGCGAGCTGGCTAAAACTGGA 3' for antisense strand primer, respectively. The PCR product was digested with *Hin*dIII and *Sph*I, then fused to the thymidine kinase promoter of PBLCAT2 (Luckow and Schutz, 1987) to yield JCVEnh/CAT2.

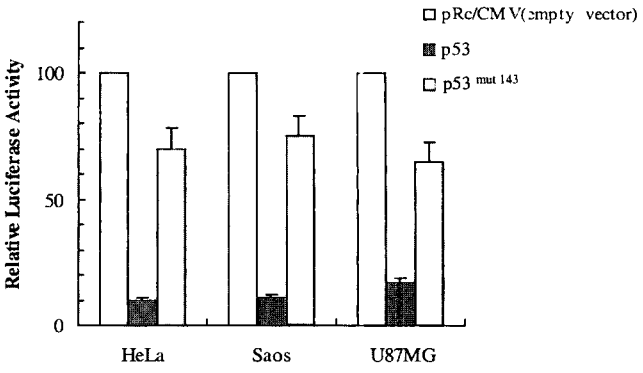
Used as effector plasmids were pJC-T, which expressed full-length JC virus large T antigen and pCMV-p53 and pC MV-p53mut143, which expressed wild type and mutant p53 protein, respectively, as described previously (Henson *et al.*, 1995; Bargonetti *et al.*, 1991).

## RESULTS AND DISCUSSION

### p53 Represses the JC virus promoter activity

Previous observations have demonstrated that p53 interacts with many viral gene products and regulates their expression (Levine, 1990; Werness *et al.*, 1990). In the case of SV40, p53 binding sites have been identified

in the enhancer region of the promoter and the binding was inhibited by SV40 T antigen (Bargonetti *et al.*, 1991). Since the promoter of MH1 type JC virus has a structural similarity with that of SV40 (Fig. 1), we tested whether p53 regulates JC virus transcription. By coexpression of MH1 long-luc reporter gene with p53 effector plasmid, p53 was found to reduce the transcriptional activity about 10-fold in HeLa cells (Fig. 2). In contrast, the ala (143) mutant of p53, which features amino acid substitution in the middle of the DNA binding domain, decreased the JC virus promoter activity about 1.5-fold. The same extent of repression was also observed in p53-deficient Saos human osteosarcoma cells. In U87MG glioma cells where the MH1 promoter directed expression 30-fold stronger than in nonglial cells, p53 repressed JCV transcription about 5- to 6-fold; a degree of repression lower than nonglial cells (Fig. 2). These results show that p53 represses JC virus promoter activity regardless of cell types.




| | HeLa | Saos | U87MG |
|---|---|---|---|
| - | 2688 | 911 | 82532 |
| pRc/CMV | 2611 | 870 | 77349 |
| P53 | 250 | 95 | 13164 |
| p53 mut 143 | 1827 | 652 | 50276 |

**Fig. 2.** p53 repressed JC virus promoter activity. Transfection was performed using pRc/CMV, pCMV-p53 and pCMV-p53mut143 as expression plasmids. pRc/CMV was used as the empty control plasmid and pCMV-53mut143 as a binding domain mutant of p53. The cells were harvested 48h after transfection, and luciferase expression was measured and normalized to β-galactosidase activity. The luciferase activities are presented in the table (average values of three experiments). The normalized luciferase activity driven by MH1long-luc in each cell lines was set to 100 to allow comparison of the effect of p53 and these relative activities are presented in the graph. p53 reduced the transcriptional activity about 10-fold in nonglial cells (HeLa & Saos cells) and 6-fold in U87MG glioma cells. However, coexpression of p53 mutant did not repress the promoter as much as wild type p53.

## Enhancer region is important for p53-mediated repression

To identify cis-regulatory elements responsible for p53-mediated repression, several mutants were created in the basal promoter region and cotransfection assay was performed using p53 expression plasmids. Previously, pentanucleotide, TATA and Sp1 sites downstream of TATA have been shown to be critical for T antigen-induced transactivation (Kim *et al.*, 2000). Because T antigen can interact with p53, we tested whether the above basal promoter mutants affected p53-mediated repression. Unexpectedly, none of the mutations produced a significant change in the p53-mediated repression (Fig. 3). With deletion of the enhancer region, the extent of repression decreased from 6.5 -10 fold to 2 - 2.5 fold. These results indicate that the enhancer region, rather than the basal promoter region, is important for p53 activity. These results are somewhat related with the previous findings that the p53 binding site on the SV40 promoter resided in the enhancer region (Bargonetti *et al.*, 1991). The fact that deletion of the enhancer region did not completely block p53-mediated repression suggests that protein-protein interaction between p53 and other transcription factors might be involved in this regulation.

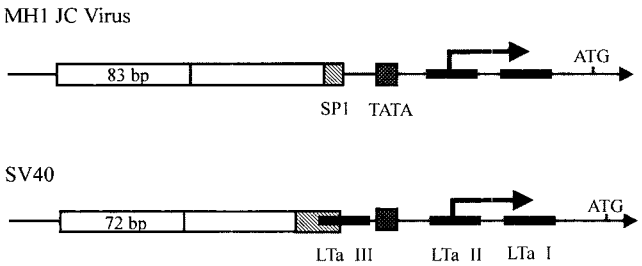



**Fig. 1.** Schematic of the MH-1 JC virus and SV40 early promoters. Open boxes indicate direct tandem repeats of the indicated number of base pairs, dotted boxes represent TATA homologies, and striped boxes represent Sp1 binding sites upstream of the TATA sequence. The SV40 promoter contains three binding sites for the viral protein large T antigen (black boxes), whereas the JC virus early promoter contains only sites LTa I and II, based on sequence homology with SV40 promoter.

## Enhancer region confers p53-mediated repression to a heterologous promoter

To verify the importance of the enhancer region for p53-mediated repression, we synthesized the enhancer region by PCR and cloned in front of the HSV thymidine kinase promoter of PBLCAT2 to produce JCVEnh/CAT2 (Fig. 4). After cotransfection of JCVEnh/CAT2 plasmid with p53,

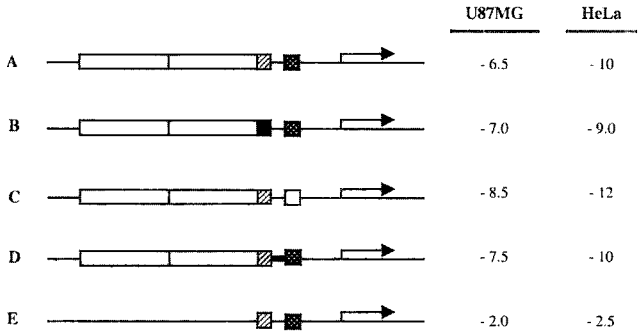
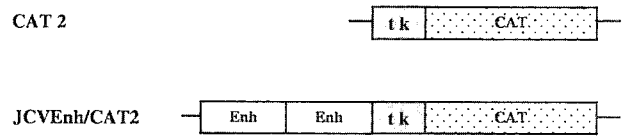



**Fig. 3.** Deletion of the enhancer region diminished p53-mediated repression, whereas base substitutions of transcription factor binding sites in the proximal promoter region did not produce a significant change. After cotransfection of the reporter gene with p53 expression plasmid, luciferase expression in the presence of p53 was divided by its expression in the absence of p53. Therefore, negative values represent repression of the JC virus promoter. Promoter A is the wild-type MH1 promoter, B contains mutations in the Sp1 binding site, C contains mutations in the TATA sequence to an irrelevant sequence, D has mutations across the pentanucleotide sequence, and E lacks the upstream enhancer sequence.




| | U87MG | | | HeLa | | |
|---|---|---|---|---|---|---|
| | - p53 | + p53 | Ratio | - p53 | + p53 | Ratio |
| CAT 2 | 6739 | 6126 | - 1.1 | 6894 | 2298 | - 3.0 |
| JCVEnh/CAT2 | 7403 | 2007 | - 3.7 | 5258 | 876 | - 6.0 |

**Fig. 4.** The enhancer confers p53-mediated repression to the heterologous thymidine kinase (TK) promoter. **Upper panel:** schematic illustration of reporter gene constructs. The enhancer element was cloned in front of the TK promoter to produce JCVEnh/CAT2. After co-transfection of these plasmids with p53 into HeLa or U87MG cells, the CAT activity(cpm) driven by each construct was normalized by the β-galactosidase activities. **Lower panel:** The CAT activities are mean values from four or five independent samples.

CAT activity was compared. In U87MG glioma cells, p53 decreased JCVEnh/CAT2 activity about 3.7 fold, whereas it did not affect CAT2 activity. In HeLa cells, JCVEnh/CAT2 activity was decreased about 6-fold by p53 whereas CAT2 activity was decreased 3-fold, indicating that p53 also repressed the thymidine kinase promoter activity in HeLa cells. However, changes in the extent of repression became larger following the addition of the enhancer

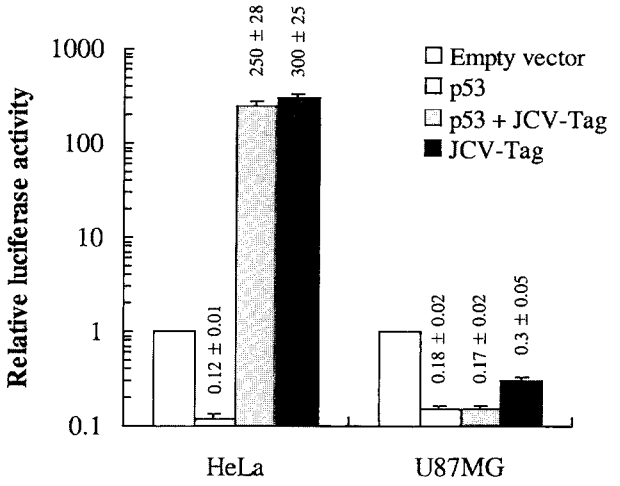



**Fig. 5.** T antigen de-repressed p53 activity on the JC virus promoter in non-glial cells but not in glial cells. Cotransfection was performed using MH1long-luc reporter plasmid, p53 and T antigen expression plasmid, in a molar ratio of 1 : 0.1 : 0.1, into HeLa and U87MG cells. Cells were harvested 48 h after transfection and luciferase activities were measured and then normalized by protein concentration. The luciferase activity driven by MH1long-luc itself was set to 1.0 to allow comparison of repression or transactivation by p53 or T antigen, respectively. Relative luciferase activity by p53 or T antigen cotransfection is presented as mean ± SEM values from six to eight samples on a logarithmic scale.

region. Although a major role of the enhancer is to increase the promoter strength of JC virus, the enhancer did not increase thymidine kinase promoter activity. From these results, we confirm that the enhancer region is important for p53-mediated repression in the context of homologous and heterologous promoters. Bargonetti *et al.* (1991) identified p53 binding in the enhancer region of the SV40 promoter by DNase I footprinting analysis, although the enhancer region did not contain consensus p53 protein binding sequences. To address whether p53-mediated transcriptional repression is caused by direct p53 binding to the JC virus promoter or by protein-protein interactions, DNase I footprinting or gel shift assay using recombinant p53 protein would be required.

## T Antigen de-repressed p53 activity on the JC virus early promoter

We have previously demonstrated that T antigen controls the JC virus basal promoter in a glial cell-specific manner, since T antigen repressed the JC virus and SV40 early promoter in glioma cells but induced strong activation of the JC virus promoter in nonglial cells. Henson *et al.* (1995) showed that p53 and T antigen were physically associated in U87MG cells after cotransfection of pJC-T and pCMV-p53. This result suggests that T antigen may regulate p53

activity. To explore this possibility, the expression plasmids of p53 and JC virus T antigen were cotransfected with the MH1 reporter plasmid. In HeLa cells, T antigen abolished p53-mediated repression and increased transcription activity about 250-fold, an extent comparable to the changes induced by T antigen only (Fig. 5). In contrast, T antigen did not produce any significant change in p53-mediated repression in U87MG cells. We also tried cotransfection in Saos cells and obtained results similar to those in HeLa cells. These results suggest that T antigen regulates not only the basal transcriptional activity but also the p53-mediated repression of the JC virus promoter in a cell-specific manner. In addition, transcriptional activation of MH1 JC virus early promoter by T antigen in nonglial cells implies the presence of silencers(Henson *et al.*, 1995; Kim *et al.*, 2000). Therefore, the finding that T antigen de-repressed p53 activity in nonglial cells suggests that p53 might be a candidate silencer protein that represses JC virus expression in nonglial cells. The results also provide the evidence for the possible correlation between glial cell-specific expression of JC virus and generation of PML and brain tumors in glial cells.

In conclusion, we demonstrated that p53 acts as a strong repressor to the JC virus promoter and that it thus has therapeutic potential for PML and human tumors caused by JC virus.

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