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Mapping 5′ Termini of JC Virus Early RNAs

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Within its enhancer promoter region, the MAD-1 strain of JC virus (JCV) has two 98-base-pair tandem repeats, each containing a TATA box-like sequence. In the present study, polyadenylated early JCV mRNAs were isolated 5 or 29 days after infection of primary human fetal glial (PHFG) cells. By using S1 nuclease, the 5′ termini of the early mRNAs were mapped to nucleotide position(s) (np) 122 through 125, which lies within an AT rich region (at np 113 through 127). In contrast, when JCV DNA was transcribed in vitro, we observed a single major cluster of 5′ start sites at np 94 through 97, which is approximately 25 base pairs downstream from one of the TATA boxes. By day 5, the earliest time at which JCV RNA was detected, viral DNA replication had begun; it continued for at least an additional 20 days. Since more late than early RNA was present at 5 days postinfection, the early RNAs whose synthesis began at np 122 through 125 may be analogous to SV40 late early mRNA (Ghosh and Lebowitz, J. Virol. 40:224–240, 1981). However, we have not detected RNAs with 5′ termini 25 to 30 bp downstream from the TATA box at earlier times. While JCV contains two identical TATA boxes, one in each of the 98-bp repeats, only the upstream TATA box functions as an early promoter element.

JC virus (JCV) is a human papovavirus that is the etiologic agent of the fatal demyelinating disease, progressive multifocal leukoencephalopathy (21, 22). In contrast to simian virus 40 (SV40) and BK virus, which can be grown in numerous cell types (24), JCV can be grown in vitro only in human fetal glial cells (20). In addition, JCV has a more prolonged life cycle compared with the other papovaviruses, requiring 3 to 4 weeks in vitro to produce significant viral titers in primary human fetal glial (PHFG) cells (20, 24).

The difficulty of obtaining and growing PHFG cells has hampered the study of JCV, so that relatively little is known about the molecular biology of this interesting virus. The MAD-1 strain of JCV has been sequenced (6) and shown to contain two 98-base-pair (bp) tandem repeats located in a position analogous to that of the SV40 72-bp repeats. Each of the JCV 98-bp repeats contains the TATA box-like (M. Goldberg, Ph. D. thesis, Stanford University, Stanford, Calif., 1979) sequence TATATAT within it. We have shown that these JCV tandem repeats possess an enhancer function that is tissue specific for human fetal glial cells (11). Although the start site for JCV early RNA made in transformed hamster cells has been approximately mapped (5), the JCV early mRNA start sites used during a lytic cycle have not been precisely determined, and the JCV early promoter has not been defined.

JCV RNAs have been isolated from infected cells or after in vitro synthesis. To identify their 5′ endpoints, JCV RNAs were hybridized to a 582-nucleotide-long DNA probe that was 5′ ^{32}P end labeled at nucleotide position (np) 4960 (Fig. 1). The DNA-RNA hybrids were digested with S1 nuclease (3), and the digestion products were then separated on a 6% acrylamide-urea gel (Fig. 2). Subsequently, the 5′ termini of the in vitro transcribed RNAs were mapped more precisely by running these digestion products on a high-resolution gel with standards prepared by base-specific chemical cleavage (16) of the DNA probe (Fig. 3).

The RNA synthesized in the HeLa cell extract had one major 5′ terminus that mapped to np 94 through 97, or approximately 25 bp downstream from the upstream TATA box (Fig. 2, lane 6; Fig. 3). The in vitro RNA also contained a very minor transcript whose 5′ end mapped to the upstream AT-rich stretch.

The polyadenylated early mRNA isolated from JCV-infected PHFG cells on day 5 had one major 5′ terminus, but this was located within the upstream AT-rich stretch (Fig. 2. lane 3). RNA isolated from infected PHFG cells on day 29 gave an identical result (Fig. 2, lane 5). When we decreased the concentration of S1 nuclease enzyme and lowered the temperature of digestion, the 5′ termini still mapped within the upstream AT-rich stretch (data not shown).

Since preliminary experiments indicated that the major 5′ start site of the in vivo transcripts appeared to lie within a long AT-rich stretch, we worried about an artifact due to the ability of the S1 nuclease to cleave duplex molecules in AT-rich regions. Therefore, we cloned the 880-bp *Stu*I-*Stu*I fragment of JCV DNA (np 4960 to 710) into a vector containing an upstream T7 polymerase promoter (Genescribe, United States Biochemical Corp.). The orientation of insertion was such that transcription of the JCV DNA from the T7 polymerase promoter would yield RNA containing early JCV mRNA sequences. This T7-transcribed JCV RNA was hybridized in parallel to the same DNA probe used to analyze the JCV-infected PHFG cell RNA, and the hybrids were then treated with S1 nuclease under identical conditions. Since the T7-transcribed JCV RNA spans the entire length of the DNA probe, any digestion products other than full-length probe must be due to an S1 nuclease artifact.

We found that the T7-transcribed JCV RNA hybridized and digested under conditions identical to those used with in vivo RNA permitted a small amount of artifactual cutting in the two AT-rich stretches (in addition to the expected full-length probe protection). However, the proportion of protected DNA fragments mapping to the upstream AT-rich stretch was much smaller than that seen with the in vivo RNA (Fig. 2, cf. lane 4 with lanes 3 and 5). In addition, the T7 RNA showed equal amounts of artifactual cutting for both of the AT-rich stretches, whereas hybrids formed with the in vivo RNA were cleaved exclusively in the upstream AT-rich stretch. Therefore, we conclude that the 5′ terminus of JCV early mRNA lies within, or possibly very near to, the upstream AT-rich stretch. We have mapped the two S1 cleavage products of the T7 RNA more precisely by analysis

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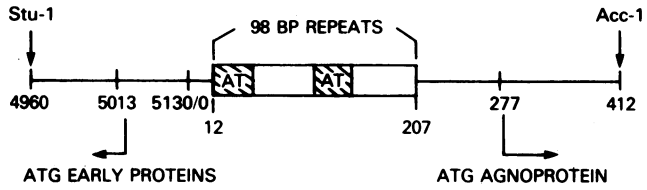

FIG. 1. Preparation of the 5′ ^{32}P-end-labeled DNA probe. The genomic locations of the JCV 98-bp tandem repeats and AT-rich stretches is shown, with the numbering system of Frisque (6). The DNA probe used for S1 mapping studies was 5′ ^{32}P end labeled at *Acc*I (np 412), and then the 582-bp *Acc*I-*Stu*I fragment was isolated by gel electrophoresis.

on a sequencing gel like that shown in Fig. 3 (data not shown). This showed the upstream cleavage site to be located at np 122 through 125, a position identical to that observed with the in vivo RNA.

When SV40 early mRNA start sites used before DNA replication were mapped, the major 5′ start sites were located 25 to 30 bp downstream of the single SV40 TATA box (2, 7). However, after DNA replication, the major SV40 early mRNA start site is within, or slightly upstream of, the AT-rich stretch containing the TATA box (4, 8, 9). Since the JCV early RNA 5′ start site is in a position analogous to that of the SV40 late early start site, we wondered whether the virus had already switched to the late phase of its life cycle at day 5, the earliest time that we could detect any viral RNA. We attempted to analyze RNA obtained from infected PHFG cells on day 3, but, due to a limitation in the quantity of PHFG cells available, we were unable to obtain sufficient RNA for S1 analysis.

As an alternative approach, we next studied the kinetics of JCV DNA replication in PHFG cells. Multiple dishes (100 mm^2) of PHFG cells were simultaneously infected with 100 hemagglutinin units of MAD-1 JCV. The JCV DNA from duplicate plates of PHFG cells was prepared by using the Hirt supernatant technique (10) on days 0, 1, 3, 5, and 17 of JCV infection. The DNA was denatured and fixed onto a nitrocellulose filter as previously described (17). The filter was then hybridized overnight in 50% formamide (23) with a [^{32}P]UTP-labeled RNA probe. This RNA probe was transcribed by T7 RNA polymerase from the same plasmid described above. The filter was washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) three times, treated with RNAse, and then exposed to film. The amount of JCV DNA decreased to a nadir on day 3 postinfection (Fig. 4A). At day 5, the quantity of JCV DNA was only slightly greater than at day 3, but by day 17, the JCV DNA had clearly replicated.

In addition to the above study, duplicate plates of JCV-infected PHFG cells were pulse-labeled with [^{3}H]thymidine (100 μCi/ml of medium) for 2 h before extracting the DNA by the Hirt supernatant technique. The DNA was then hybridized 5 days in 50% formamide to nitrocellulose filters containing denatured JCV-pBR322 DNA. The filters were washed in 0.1× SSC three times, and then the amount of tritium bound to each filter was determined by scintillation counting (Fig. 4B).

No JCV DNA replication, as detected by [^{3}H]thymidine incorporation into JCV DNA, was observed on days 0, 1, and 3. By day 5 of infection, JCV DNA replication could be detected, but its rate was still much less than that seen at day 15.

As further evidence that by day 5, the JCV infection had switched to the late phase of the life cycle, we compared the amount of late versus early JCV RNA present at different times in the JCV-infected PHFG cells. By inserting the *Stu*I-*Stu*I fragment of JCV (np 4960 to 710) in the opposite orientation in the vector containing the T7 polymerase promoter, we were also able to make a [^{32}P]UTP labeled RNA probe that hybridized specifically to late JCV RNA (Fig. 5). At day 3 of infection, the amount of both early and late JCV RNA was very low. However, by day 5 of infection, there was already more late RNA than early RNA,

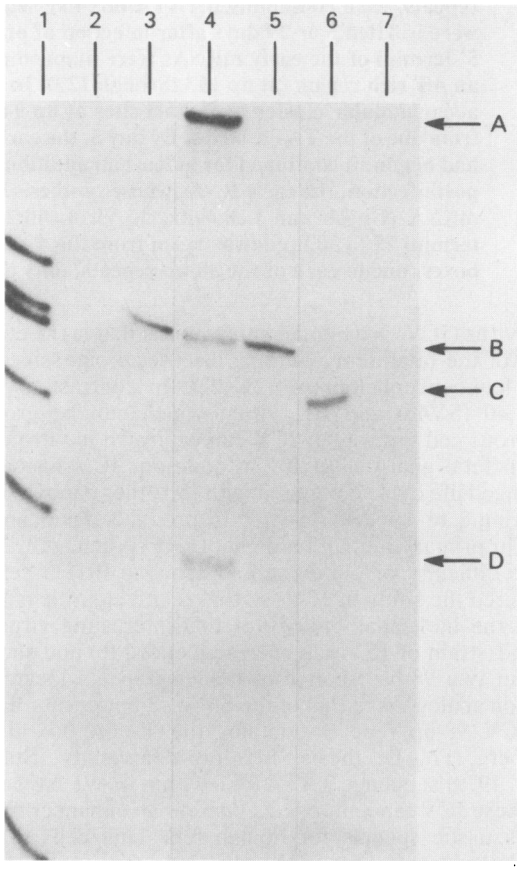

FIG. 2. Mapping the 5′ termini of JCV early mRNA. The primary human fetal glial cells were obtained and grown essentially as described by Padgett et al. (20). A stock of MAD-1 JCV was prepared by transfecting PHFG cells with DNA from a plasmid originally made by inserting MAD-1 DNA into pBR322 at the *Bam*HI site. The plasmid, a gift of Peter Howley, was cut and religated to produce intact JCV DNA before transfection. To prepare in vivo JCV RNA, flasks (75 cm^2) of PHFG cells were each infected with 200 to 400 hemagglutinin units (18) of JCV, and the cells were harvested 5 days later. Polyadenylated RNA was then isolated as described by Kenney et al. (10a). JCV RNA was hybridized to a DNA probe 5′ ^{32}P end-labeled at the *Acc*I site (np 412), digested with S1 nuclease (5,000 U/ml at 23°C) for 1 h, and then electrophoresed on a 6% acrylamide–urea gel (3, 16). Lane 1, DNA molecular weight marker (sizes are 310, 281, 271, 234, 194, and 118 nucleotides); lane 2, no RNA (control); lane 3, day 5 in vivo RNA; lane 4, T7 polymerase-transcribed in vitro RNA; lane 5, day 29 in vivo RNA; lane 6, HeLa extract in vitro RNA; lane 7, HeLa extract in vitro RNA made in the presence of α-amanitin (1 μg/ml). Arrow A, Position of the full-length DNA probe; arrow B, position of DNA cleaved at the upstream AT-rich stretch; arrow C, position of DNA cleaved at the in vitro RNA 5′ terminus, located at np 94 through 97 (see Fig. 3); arrow D, position of DNA cleaved at the downstream AT-rich stretch.

indicating that the virus had switched to the late phase of infection. The ratio of late RNA to early RNA at day 5 was similar to that seen on day 17 (data not shown).

Our results indicate that after day 5 of infection, the MAD-1 strain of JCV grown in PHFG cells had one major 5′

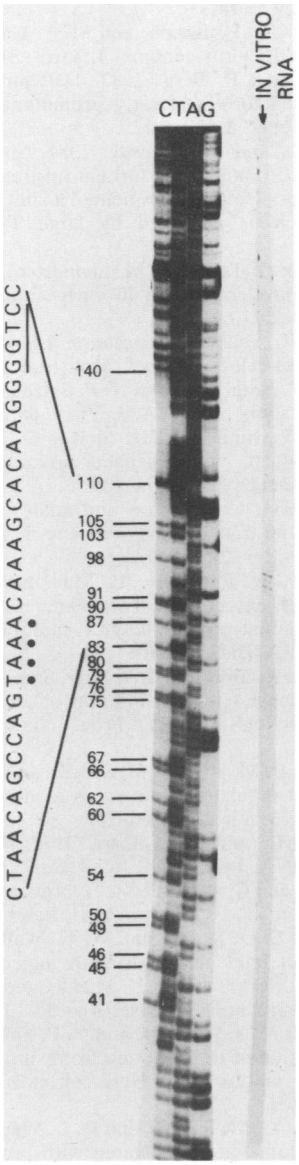

FIG. 3. Mapping of 5′ termini of JCV RNA transcribed in vitro. We prepared in vitro transcripts of JCV DNA by using a Manley HeLa cell extract (14) under conditions previously described (19; Kenney et al., submitted). The 5′ termini of RNA transcribed in vitro were more precisely determined by electrophoresis of the S1 digestion products next to a sequencing ladder generated by chemical cleavage (16) of the DNA probe. Numbers show the genome locations of the bands in the C ladder. The letters to the left of the gel show the nucleotide sequence from np 83 to 110. The nucleotides with dots underneath them are the positions of the 5′ termini of the in vitro-transcribed JCV early RNA. The four nucleotides underlined (GGTC) were not present in the MAD-1 DNA sequenced by Frisque (6) but were present in our DNA. Because of the position of these four extra nucleotides, the MAD-1 DNA used here actually has two 102-bp tandem repeats. To diminish confusion, these have been referred to as 98-bp repeats in the text.

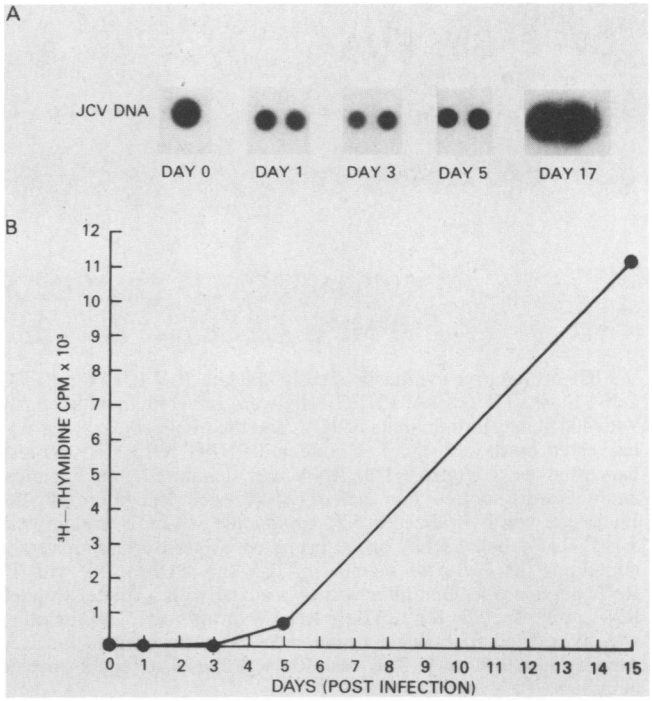

FIG. 4. JC virus replication in primary human fetal glial cells. PHFG cells in dishes (100 mm^2) were infected with 100 hemagglutinin units of JC virus. (A) JCV DNAs were obtained separately as Hirt supernatants (10) from duplicate plates on days 0, 1, 3, 5, and 17 of infection. One tenth of the total volume of each Hirt supernatant was denatured, spotted on a nitrocellulose filter (17, 23), and hybridized overnight in 50% formamide at 60°C to the single-stranded [^{32}P]UTP-labeled RNA probe synthesized by T7 polymerase. After being washed three times in 0.1× SSC and being treated with RNAse, the filters were exposed overnight at −70°C with an intensifying screen. Each spot (days 1, 3, 5, and 17) represents DNA from a separate dish of infected cells. (B) Duplicate plates of JCV-infected PHFG cells were pulse-labeled with [^{3}H]thymidine (100 μCi/ml of medium) for 2 h before a Hirt supernatant (10) was prepared on days 0, 1, 3, 5, and 15 of infection. One fourth of the ^{3}H-labeled JCV DNA in the supernatant was hybridized for 5 days in 50% formamide at 42°C (17, 23) with nitrocellulose filters containing denatured JCV-pBR322 DNA. The filters were washed in 0.1× SSC three times, and then the amount of ^{3}H on each filter was determined by scintillation counting. The numbers on the bottom of the graph indicate the day of JCV infection.

start site for early mRNA which is located within, or slightly upstream of, the AT-rich stretch located at np 113 through 127. Since by day 5, the JCV DNA has begun replication, and the amount of JCV late RNA already greatly exceeds the amount of early RNA, the 5′ start site mapped here is analogous to the SV40 late early mRNA start site (4, 8, 9). This SV40 start site is also located within, or slightly upstream of, an AT-rich stretch. Whether JCV, like SV40, also has a 5′ start site located 25 to 30 bp downstream from a TATA box during the earlier stage of infection is not known; we were unable to obtain sufficient quantities of JCV RNA at day 3 of infection to examine this possibility.

Since the MAD-1 strain of JCV contains two identical copies of the 98-bp sequence that includes the TATA box, it is perhaps surprising that only the upstream 98-bp repeat appears to function as an early promoter, particularly since the unique TATA box present in other strains of JCV lies in a position analogous to the downstream TATA box of MAD-1 JCV (15). It seems likely that the sequence upstream

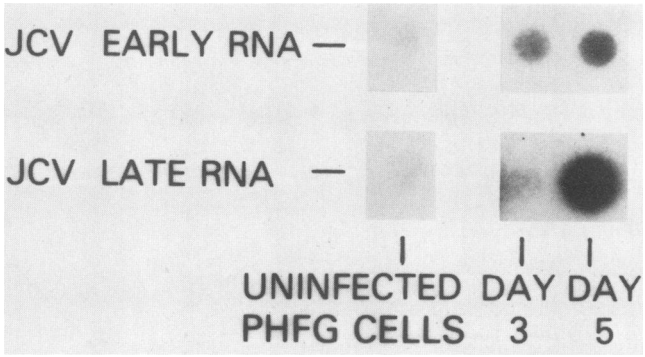

FIG. 5. Relative synthesis of early and late JCV RNAs in PHFG cells. Flasks (75 cm^2) of PHFG cells were infected simultaneously with 400 hemagglutinin units of JCV, and the total cellular RNA was harvested on days 3 and 5. Uninfected PHFG cell RNA was also harvested as a control. The RNA was denatured, and identical amounts were spotted onto each of two nitrocellulose filters (17, 23). Each filter was hybridized in 50% formamide at 60°C overnight with a [^{32}P]UTP-labeled RNA probe prepared by transcribing a vector containing the *StuI-StuI* segment of JCV (np 4960 to 710) with T7 RNA polymerase. One filter was hybridized with a single-stranded RNA probe specific for JCV late RNA (bottom row), and the other was hybridized to a single-stranded RNA probe specific for JCV early RNA (top row). The two RNA probes had equal specific activities.

of the 98-bp repeats plays a role in the choice of the upstream start site.

One consequence of placing the early mRNA 5′ start site within the upstream repeat is that the AUG codon beginning at np 64 could initiate synthesis of a protein of 37 amino acids in length, which would not be possible if the 5′ start site were positioned within the downstream 98-bp repeat. Although this putative JCV early protein has yet to be identified, the ATG at np 64 is surrounded by sequences evaluated as moderately favorable for ribosomal translation by the criteria of Kozak (12). Alternatively, the upstream ATG may be bypassed with translation beginning at the downstream ATG located at np 5013.

Our DNA replication studies are in agreement with in situ hybridization studies of JCV-infected PHFG cells by Major et al. (13), who found that most JCV DNA did not replicate until the third week of infection. This delay in the onset of an appreciable amount of JCV DNA replication was not due to a requirement for several cycles of infection (i.e., spread of virus from cell to cell), since the majority of PHFG cells already express JCV T antigen by day 7 (13, 21) and viral titers were not substantial before the third week of infection. The long duration of the JCV life cycle is in contrast to other papovaviruses and will be an interesting area for further study.

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