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Effect of Primer Selection on Spuriously Generated Deletion of a Tandem Repeat during PCR

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In general, the PCR faithfully amplifies the DNA fragment contained between unique primer binding sites. However, we have found that priming at certain sites can give rise to an artifactual deletion of one copy of a tandem repeat contained within the region to be amplified.

The human polyomavirus JC (JCV) is a 5130-bp circular DNA virus that circulates widely in the population and frequently establishes latency in the kidney. Rarely, JCV causes a fatal central nervous system (CNS) infection known as progressive multifocal leukoencephalopathy (PML) in immunocompromised patients. JCV DNA is divergently transcribed.[1] The noncoding regulatory region containing the origin of DNA replication and transcriptional promoter/enhancer sequences is located between the early (large and small T-antigens) and late genes (capsid proteins).[1] Brain "adaptation" of the virus requires rearrangement of the "archetypal" regulatory region of the kidney form of the virus by a process of deletion and duplication.[2] In JCV (Mad-1), the prototype strain, a 98-bp promoter/enhancer is repeated in tandem to the late side of the origin (positions 12–109 and 110–207).[3] This regulatory region structure is one of the many variants subsequently isolated from PML brains in which tandem repeats appear to be generated by deletion and duplication of the archetypal sequence found in the kidney. While amplifying the JCV (Mad-1) regulatory region by PCR, we observed that one primer pair produced an unexpected deletion of one of the 98-bp repeat units.

PCR was carried out with 1–2 pg of pBR322 cloned JCV (Mad-1) DNA (used as positive control) or 10–25 μl of brain tissue extracts. The brain tissue extracts were prepared as follows. Frozen brain sections (10-μm thick) were digested for 2–4 hr at 56°C in 100 μl of buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 0.45% (vol/vol) Tween 20, 0.45% (vol/vol) Nonidet P-40, and 100 μg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO). Proteinase K was then inactivated by heating at 95°C for 15 min. Samples were centrifuged and the supernatant was used for PCR. To increase the sensitivity of the reaction, the samples were subjected to nested PCR and compared with nonnested PCR. Nested PCR was carried out with outer primers JRR17/JRR18 (Table 1) for the first 15 cycles, after which inner primers (JRR1/JRR8 or JRR7/JRR8) were added and PCR continued for another 40 cycles. Nonnested PCR was carried out using only the inner primers for 40 cycles of amplification.

A 327-bp fragment was amplified using JRR1 and JRR8 in both nested (Fig. 1A, lane 1, upper band) and nonnested PCR (Fig. 1A, lane 3, upper band). In addition to this expected band, a lower band was observed that was relatively more intense in the nested PCR (lane 1). The lower band was much stronger relative to the upper band when nested PCR was performed using brain tissue extracts (Fig. 1B). In one tissue sample (Fig. 1B, lane 2), the lower band was the predominant amplification product. The reason for the difference observed between amplification of plasmid DNA and the brain extracts is not clear. Apparently, the presence of cellular DNA or other components of the brain extract contributes to the increased amplification of the lower band.

Cloning and sequence analysis of the upper and lower bands was performed following extraction of each band from the gel. The upper band was shown to be the expected 327-bp product containing both 98-bp repeats, while the lower band in Figure 1, A and B, completely lacked one of the repeats and was therefore 229

TABLE 1 Primer Sequences for JCV Regulatory Region Amplification

Primer	Position[a]	Sequence (5′ → 3′)	Fragment size
JRR1	(5100–5124)	CTACTTCTGAGTAAGCTTGGAGGCG	JRR1/8 = 327 bp
JRR7[b]	(4979–5009)	TCCATGGATCCCTCCCTATTCAGCACTTTGT	JRR7/8 = 454 bp
JRR8	(296–266)	GACAGCTCGAGAAGAACCATGGCCAGCTGGT	
JRR17	(4921–4942)	TCTCATGACAGGAATGTTCCCC	JRR17/18 = 534 bp
JRR18	(324–303)	TTTACTAACTTTCACAGAAGCC	

[a]Nucleotide positions in the JCV genome from ref. 3.
[b]JRR7 and JRR8 were altered to introduce *Bam*HI and *Xho*I restriction sites, respectively, near their 5′ ends (underlined).

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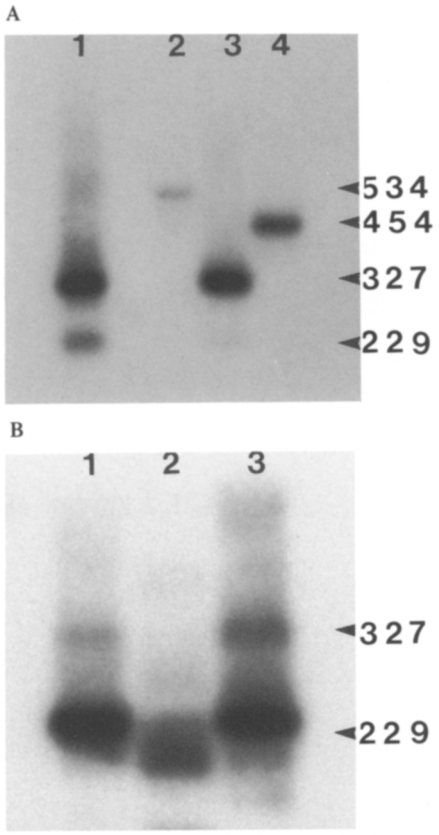

FIGURE 1 Southern analysis of PCR products following electrophoresis on a 1.5% agarose gel. PCR reaction mix in 100 μl contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM dNTPs, and 0.5 μM each primer. After an initial denaturation at 94°C for 5 min, 2.5 units of Cetus *Taq* polymerase were added and PCR was performed in a Perkin-Elmer Cetus thermal cycler (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, with a final extension at 72°C for 10 min). Both blots were probed with ^{32}P end-labeled oligonucleotide probe (5′-ATACCTAGGGAGCCAACCAGCTAACA-3′) (positions 63–88 and 161–186 in first and second repeats, respectively). (*A*) (Lanes *1* and *3*) Nested (JRR17/18 and JRR1/8) and nonnested (JRR1/8) PCR, respectively, with plasmid DNA; (lane *2*) PCR with JRR17/18 using a tissue extract; (lane *4*) nested PCR (JRR17/18 and JRR7/8) with a tissue extract. (*B*) (Lanes *1–3*) Nested PCR with JRR17/18 and JRR1/8 using three brain tissue extracts. Numbers on the right indicate the sizes of the fragments in base pairs.

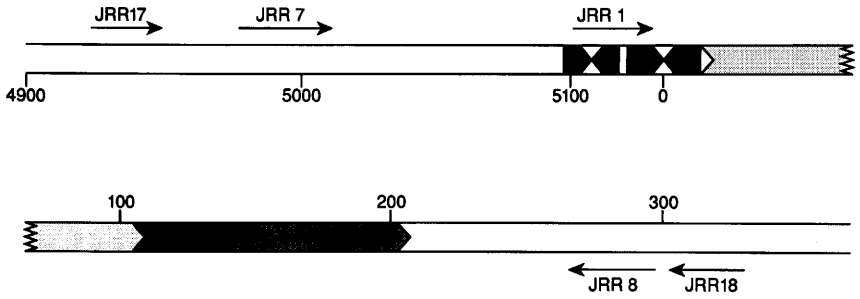

FIGURE 2 Location of the primer binding sites in the regulatory region of JCV Mad-1. Light and dark stippled bars indicate the positions of 98-bp tandem repeats. Black pointed boxes near the origin represent the palindromes with potential for secondary structure.

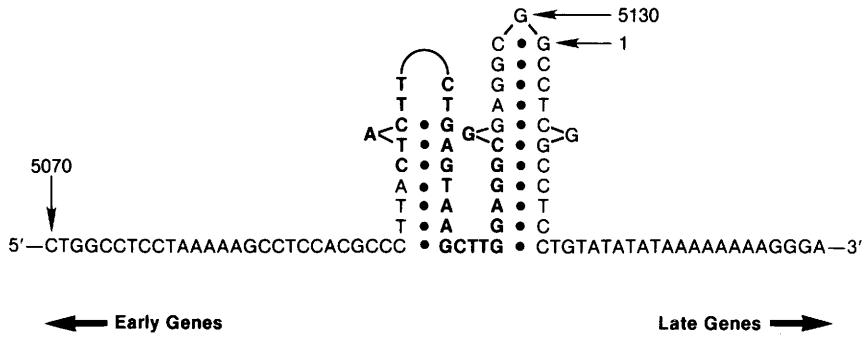

FIGURE 3 Potential secondary structure encompassing the region containing the origin of DNA replication.[4] Note that the JRR1 primer binding site (bold letters, positions 5100–5124) is a part of both hairpin loops.

bp in length, exactly 98 bp shorter than the upper band.

The generation of the deleted lower band could be abolished when the JRR1 primer was replaced by JRR7 in the nested PCR (Fig. 1A, lane 4). Moreover, when JRR7/JRR8 or JRR17/18 were used alone in a nonnested PCR, the lower band did not appear (Fig. 1A, lane 2 shows the band obtained with JRR17/18).

Primers JRR7 and JRR17, which do not promote generation of the lower band, are located farther upstream from the 98-bp repeat unit than is JRR1 (Fig. 2). Therefore, we suggest that the close proximity of primer JRR1 to the first repeat unit (only 17 bp upstream) allows some of the product that is partially extended into the repeat unit to dissociate during the first few cycles of PCR. Reannealing of this incomplete product within the second repeat unit during a subsequent cycle falsely primes a second extension reaction. Further extension produces a deleted product lacking one 98-bp repeat unit that is preferentially amplified in later cycles.

It may also be significant that the JRR1 site (positions 5100–5124) encompasses the core sequence of the origin of replication (positions 5096–12) (Fig. 3). The primer binding site overlaps two palindromic regions that have potential for internal secondary structure.[4] If this secondary structure actually forms, it could destabilize JRR1 primer binding and thereby promote dissociation of the partially extended product as suggested above.

Our data indicate that tandem repeats can be subject to deletion artifacts during PCR amplification if one primer site is close to the repeat unit and/or the primer binding site encompasses a region of potential secondary structure. In the case of repeated viral promoter/enhancer elements, these spurious products should not be confused with archetypal regulatory sequences, which may naturally lack the sequence duplication. The results emphasize the importance of primer selection in eliminating deletions spuriously generated by PCR.

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