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Genotypes of JC virus, DNA of cytomegalovirus, and proviral DNA of human immunodeficiency virus in eyes of acquired immunodeficiency syndrome patients

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JC virus (JCV) is a human polyomavirus that exists in at least eight different genotypes as a result of coevolution with different human populations all over the world. Well adapted to its host, it usually persists in the kidneys and possibly the brain. If the host becomes immunodeficient, JCV can cause the fatal demyelinating disease progressive multifocal leukoencephalopathy (PML). There is increasing evidence that JCV is transactivated by cytomegalovirus (CMV) and the human immunodeficiency virus (HIV). Both CMV and HIV can infect the retina of acquired immunodeficiency syndrome (AIDS) patients, causing severe necrosis in the case of CMV retinitis or a mild HIV-associated vasculopathy, with bleeding and cotton wool spots. The authors therefore investigated by polymerase chain reaction (PCR) whether DNA of these three viruses was detectable in paraffin-embedded eyes of AIDS patients with a clinical history of CMV retinitis. From a total of 65 eyes, JCV was detected in 21 (32%). Thirty-six (55%) were positive for CMV and 6 (9%) for proviral DNA of HIV. JCV and CMV were found in 13 eyes, JCV and HIV in 3 eyes, CMV and HIV in 1 eye, and DNA from all three viruses in 1 eye. The JCV genotypes were types 1A, 2A, 2E, 3, and 4. In 21 eyes of patients without AIDS, only one sample was JCV positive. In conclusion, JCV DNA can be detected in ocular tissue of AIDS patients at a significantly higher level than in eyes of nonimmunosuppressed patients. Further investigations will help to decide if JCV contributes to the retinopathy caused by CMV and HIV. *Journal of NeuroVirology* (2005) **11,** 58–65.

Keywords: cytomegalovirus; eye; immunodeficiency; polyomavirus; retinitis; transactivation

Introduction

Members of the family of polyomaviruses were isolated in birds, monkeys, cattle, hamster, and humans. The human polyomaviruses are JC virus (JCV) and BK virus (BKV). The transmission of JCV occurs mainly withins the family or close social environment (Kitamura *et al*, 1994). Most likely, JCV enters via the upper respiratory tract (Monaco *et al*, 1998). In the course of infection it is able to persist in the kidneys and possibly the brain (Dorries and Elsner, 1991; Tominaga *et al*, 1992). Viral DNA is detectable in the urine of 40% to 80% of individuals older than 20 years of age. In recent years, the detection of viral DNA in human tumors gave raise to question whether JCV, BKV, or the monkey polyomavirus simian virus 40 (SV40) can induce tumors not only in animals but also in humans (Arrington and Butel, 2001; Corallini *et al*, 2001; Del Valle *et al*, 2001).

JCV and BKV are named after the initials of the patients from whom they were first isolated. JCV can cause the central demyelinating disease

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progressive multifocal leukoencephalopathy (PML) (Astroem, 2001). Among acquired immunodeficiency syndrome (AIDS) patients, 4% to 7% die from this untreatable disease within months after onset of symptoms (Berger and Nath, 2001). There are no specific ophthalmic symptoms associated with PML; however, patients can develop scotomata of the visual field, according to where demyelination occurs in the optic tract (Ormerod *et al*, 1996). So far there are no reports about the presence of JCV in the human eye. BKV, however, was found to cause retinitis and meningitis in isolated cases (Bratt *et al*, 1999; Vallbracht *et al*, 1993). Hedquist and colleagues described "focal, mottled fundus pigmentation" in a 29-year-old male AIDS patient with bilateral retinitis that were later shown to be associated with necrosis, including cells positive for BKV DNA and its capsid protein VP1 (Hedquist *et al*, 1999).

In 1984, Frisque and colleagues sequenced the first complete JCV genome, named after the city of Madison in Wisconsin, JCV (Mad-1) (Frisque *et al*, 1984). JCV has a circular genome of about 5200 base pairs of double-stranded DNA, in which early genes (large T antigen, small t antigen, and T′ antigens) are transcribed counterclockwise in an early phase of viral replication. The genes for the structural proteins (VP1-3 and agnoprotein) are transcribed clockwise in the late phase of replication.

The JCV genome can vary in two ways. First, there are genotypes that coevolved with different populations worldwide. So far eight genotypes with additional subtypes were defined based on variations of the coding region sequence. JCV types 1 and 4 are mainly associated with European populations (Agostini *et al*, 1996, 2001a). Type 2 can be found in Asians but also in Native Americans of North and South America (Guo *et al*, 1998; Kitamura *et al*, 1998; Sugimoto *et al*, 1997; Agostini *et al*, 1997c). Types 3 and 6 represent different regions of Africa (Agostini *et al*, 1997a, 1998). Types 7 and 8 are located in South Asia and some Pacific Islands (Jobes *et al*, 2001; Ryschkewitsch *et al*, 2000).

The second genomic variation found in JCV is based on deletions and duplications of the viral regulatory region sequence. The transmitted form of the virus is characterized by a nonrearranged, so called "archetypal," regulatory region (Ault and Stoner, 1993; Yogo *et al*, 1990). Following infection or reactivation, the region to the late site of the origin of replication (*ori*), which contains binding sequences for transcription factors, can be rearranged by unknown cellular mechanisms (Agostini *et al*, 1997b; Newman and Frisque, 1997; Yogo *et al*, 1994).

In addition to the factors produced by the host cell, there are mechanisms by which other viruses can enhance the rate of viral replication. It is known that the human cytomegalovirus (CMV) or the human immunodeficiency virus (HIV) can transactivate JCV. In the case of CMV, it could be demonstrated that CMV supports JCV replication in nonpermissive fibroblast cell lines (Heilbronn *et al*, 1993) most likely by interaction of the immediate-early transactivator 2 (IE2) of CMV with the polyomavirus genome (Winklhofer *et al*, 2000). HIV codes for the Tat protein. Tat is a general activator for the transcription of genes in HIV. *In vitro*, it was possible to increase JCV replication with HIV Tat protein (Chowdhury *et al*, 1992; Del Valle *et al*, 2000; Raj and Khalili, 1995; Tada *et al*, 1990). This activation is mediated by the ubiquitous cellular protein Purα, which interacts with the Tat protein and binds a PUR response element similar to TAR (Krachmarov *et al*, 1996). This element is located close to the origin of replication within the regulatory region of JCV.

We therefore examined eyes of AIDS patients with a known history of CMV retinitis from the Baltimore area for the presence of viral DNA of JCV, CMV and proviral DNA of HIV. The material available was formalin-fixed and paraffin-embedded eyes from patients who had died of AIDS before the introduction of the highly active antiretroviral therapies (HAARTs). Especially however, formalin fixation produces degradation of DNA. Therefore, different methods for DNA extraction were modified and evaluated in order to optimize them for ocular tissue.

Results

DNA extraction from paraffin-embedded ocular tissue

DNA extraction was optimized by comparing two methods using 1, 5, or 10 sections of bulbi, each 4.5 μm thick. Regardless which method was used, paraffin was removed by xylol before sections were rehydrated with alcohol as described below. After digestion with proteinase K and heat inactivation of the proteinase, samples were either used in the extraction buffer for polymerase chain reaction (PCR) (method A) or further purified by chromatographic DNA isolation (method B), to remove all substances possibly inhibiting PCR (Kosel *et al*, 2001). Figure 1 shows DNA fragments with the predicted length of 110 bp or 355 bp, respectively, amplified from the β-globin gene with two different primer pairs (BEG-1 and -2; BEG-3 and -4). No PCR amplification was possible after additional chromatographic purification in all samples, most likely due to loss of DNA at the column when using only little tissue. Eyes of AIDS patients or controls were all prepared by method A. The probability of false-negative results for the detection of viral DNA was reduced by amplification of the 110-bp fragment of the β-globin gene by PCR in all samples.

Occurrence of viral DNA in eyes from AIDS patients

JCV was found in 32% (21/65 eyes) of the investigated eyes. The percentage of positive eyes in different ethnic cohorts ranged from 22% (7/32 eyes)

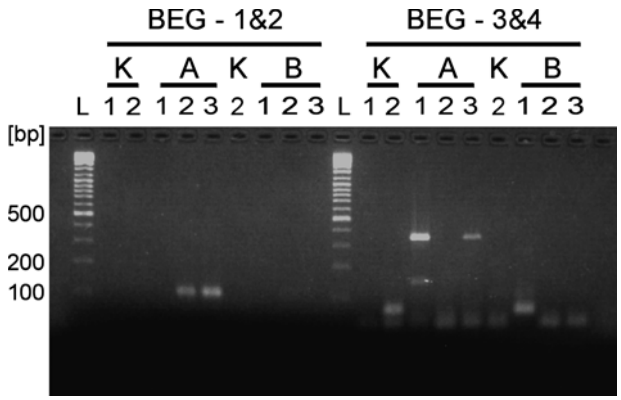

Figure 1 Gel electrophoresis of PCR-amplified fragments from the ß-globin gene using two primer pairs (110-bp and 355-bp length). Two different DNA extraction methods were used. A: proteinase K digestion + heat inactivation. B: proteinase K digestion + heat inactivation + chromatographic purification.

in the European-American group to almost double, 42% (13/31 eyes), in the African-American group (Table 1). In none of the patients were both eyes positive for JCV. Proviral DNA of HIV could be detected in 9% (6/65) of all investigated eyes. Fifty-five percent (36 of 65) of the samples tested positive for CMV DNA. In contrast to JCV, the rate of CMV identification did not differ between African (17 of 36 eyes) and European Americans (18 of 36 eyes). The age of the patients did not correlate with the occurrence of viral DNA (data not shown).

Figure 2 shows how many samples were positive for the DNA of none, one, two, or all three viruses. Overlapping circles indicate that DNA of the different viruses was found in the same eye. In 72% of all JCV-positive eyes, viral DNA was associated with CMV (57%), HIV (10%), or both (5%). DNA of all three viruses was found in the eye of a 35-year-old African-American female. Changing perspective, the majority of CMV-infected eyes was not infected by another virus at the same time. Only 13 of the CMV-positive eyes tested additionally positive for JCV and 2 for proviral HIV DNA.

JCV genotypes

From the 21 JCV strains from the eyes of the AIDS patients amplified by primers JLP-15 and -16 or JLP-1 and -4, types 4 and 2 were the most predominant, with 29% (6 of 21) each, followed by type 3 (24%, 5 of 21) and type 1B (19%, 4 of 21). The type 2 strains could be subtyped in the VP1 fragment as types 2A (5 of 6) and 2E (1 of 6). The regulatory region sequence could not be amplified from the formalin-fixed tissue sections.

Table 1 Rate of detection in eyes from AIDS patients with different ethnic background

Ethnicity	*Eyes examined*	*JCV*	*CMV*	*HIV*
African American	31	13	17	4
European American	32	7	18	2
Haiti	2	1	1	—
Total	65	21	36	6

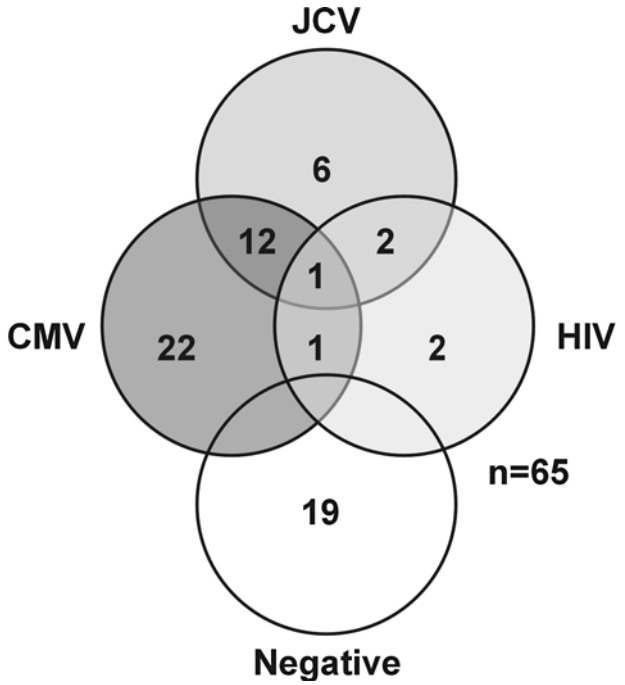

Figure 2 Viral DNA from JCV, CMV, and proviral DNA from HIV was PCR amplified from 65 eyes of AIDS patients. Each circle represents the number of samples positive for one of the viruses and those samples that were negative for all three. Overlapping circles indicate eyes with more than one virus detected in the sample. A total of 21 eyes were positive for JCV, 36 for CMV, and 6 for HIV.

JCV genotypes are based in different ethnic groups worldwide. In our cohort the highest diversity of genotypes was found among the 13 African-American patients who tested positive for JCV DNA (4× type 1A, 4× type 2A, 3× type 3, 1× type 2E, 1× type 4). In European Americans, type 4 was the most dominant followed by two type 3 strains. This JCV genotype is thought to represent an older European genotype also found in African Americans (Agostini *et al*, 2001b; Chima *et al*, 2000). Finding the African genotype, JCV type 3, in Caucasians is rather unusual (Agostini *et al*, 1995), but common in African Americans. One type 2A strain could be identified in the left eye of a 41-year-old male AIDS patient from Haiti.

When sequencing the VP1 fragment, single-point mutations were found, such as a deletion of two base pairs between 1762 and 1764 (JCV (Mad-1) numbering [Frisque *et al*, 1984]) in the left eye of a 31-year-old African American male. These mutations were not related to known typing sites and could be a result of damaged DNA due to prior formalin fixation.

JCV in control eyes

Paraffin-embedded sections of 21 eyes from patients without known immunodeficiency, known virus-induced retinopathy, or other intraocular

infections were used as controls. DNA was extracted as described (method A). All probes were tested for amplifiable DNA with the β-globulin gene before primers JLP-15 and -16 and JLP-1 and -4 were used to search for JCV. JCV DNA could be found in 1 (5%) of the 21 eyes examined. The difference in prevalence of JCV in eyes from AIDS-patients and control eyes was significant (chi square, $P < .05$).

Discussion

JCV was shown to be transactivated *in vitro* by CMV and HIV and a reverse mechanism is discussed. In patients with HIV infection as an underlying course for severe immunosuppression, CMV and HIV cause clinically visible changes in the eye, leading to severe loss of visual acuity in case of CMV. In addition, the other human polyomavirus BK was found to cause meningitis and retinitis in isolated cases (Bratt *et al*, 1999; Hedquist *et al*, 1999; Vallbracht *et al*, 1993; Voltz *et al*, 1996). This led us to the question whether JCV is involved in the clinical course of these virus-induced retinopathies. In this investigation it could be shown for the first time that JCV-DNA is present in ocular tissue. Its occurrence is significantly increased in eyes from AIDS patients (35%) when compared to a control group of immunological healthy patients (5%) without known viral disease.

For viral transactivation to take place, infection of the same tissue is a prerequisite. In the case of HIV, it could be shown that the diffusible Tat protein, an HIV regulation protein, increases JCV transcription by binding the ubiquitous cellular protein Purα, which then interacts with the PUR element in the late promotor region of JCV (Chowdhury *et al*, 1992; Daniel *et al*, 2001; Krachmarov *et al*, 1996). For CMV, the protein IE2 (immediate-early transactivator 2) activates the promotor for JCV early gene expression. This leads to an increase in T antigen and consequently to an up-regulation of JCV DNA replication (Winklhofer *et al*, 2000). With the tissue available, it was not possible to resolve this point on the cellular level. However, more than 70% of the eyes positive for JCV showed detectable DNA from CMV or proviral DNA from HIV in the same sections, indicating a possible viral interaction in a clinically relevant AIDS-associated disease. With regard to transactivation of JCV in the retina, it is of special interest that Winklhofer and colleagues observed that the presence of CMV IE2 allowed JCV replication in nonpermissive cell lines such as fibroblasts or glioblastoma cell lines. This effect was independent from regulatory region rearrangements (Winklhofer *et al*, 2000). The prevalence of HIV was unexpected low, with only 6 out of 65 eyes positive. This could be due to the fact that HIV is assumed to induce only a mild vasculopathy of the retina with small amounts of virus present. In addition, only proviral DNA of actively replicating HIV was picked up by the methods used—certainly one of the drawbacks having to use formalin-fixed paraffin-embedded tissue, which reduces the possibilities to look for intact viral RNA at a detectable level.

The JCV genome can vary in two different ways—the individual changes in the regulatory region and the genotype specific variations throughout the viral genome as a result of evolution. From the 21 JCV strains identified in the eyes of the AIDS patients, a total of five different genotypes was found. JCV genotypes are closely linked to certain populations worldwide due to coevolution of the virus with its human host and can therefore serve as a marker for human migration (Agostini *et al*, 2001b). This association of viral genotype and ethnic background of its carrier is a very useful control to exclude contamination when investigating clinical material with highly sensitive PCR methods as done in this study. The JCV genotypes determined from the 21 JCV strains in the different ethnic cohorts were mainly type 4 in the European-American group and types 1B, 2A, and 3 in the African-American group. Type 4 represents a Paleo-European variant of JCV (Agostini *et al*, 2001a). Type 3 is an African genotype of JCV, which is more closely related to Asian variants than the second African genotype, type 6 (Guo *et al*, 1996). Type 3 can be found in African Americans together with European strains 1A and 1B or Asian genotypes. Its occurrence in two of the European Americans is rather unusual but a possible result of genetic admixture as observed in African Americans (Chima *et al*, 2000), the Native American population (Agostini *et al*, 1997c), or some Asian populations (Cui *et al*, 2004; Guo *et al*, 1998). Two conclusions can be drawn from the genotyping results of JCV in eyes from AIDS patients. First, the agreement of JCV genotype and ethnic background of the patients included in this study excluded a major contamination of the samples. Second, there is no specific overrepresentation of a JCV genotype in the ocular tissue as described for PML (Agostini *et al*, 2000).

The tissue samples from eyes of deceased AIDS patients were all formalin fixed and paraffin embedded. Fixation limits the length of DNA, which can be amplified by PCR due to DNA fragmentation and PCR inhibitors. This made it necessary to optimize the DNA extraction procedure, whereas the simplest procedure of proteinase K digestion followed by heat inactivation of the enzyme proved to be the best. Additional purification of the DNA by chromatographic procedures did not enhance the sensitivity of the PCR for the β-globin gene, which was used as a positive control for DNA quality in all samples.

In summary, it could be shown that the prevalence of JCV DNA is significantly increased in ocular tissue from AIDS patients with a history of CMV retinitis, emphasising a possible transactivation of JCV by CMV or HIV as suggested earlier by *in vitro* studies. Further studies will be necessary to determine the site of infection. Possible candidates are microglial cells or astrocytes in the retina as part of the central

nervous system. Lymphocytes as carrier of JCV to the eye should be considered. To determine the configuration of the viral regulatory region will be of special interest. This will only be possible with ocular tissue not exposed to fixatives.

Material and methods

Ocular sections

A total of 65 paraffin-embedded eyes from 33 men and 2 women with AIDS and a clinical history of CMV retinitis were investigated for CMV and JCV DNA and proviral HIV DNA. Four to five unstained sections from each eye, including all layers of the retina, the uvea, cornea, and sclera, were kindly provided by Dr. Green, Wilmer Eye Institute, Baltimore, USA. Beside CMV, eyes were infected with *Toxoplasma gondii* in two cases, *Candida* in two cases, herpes simplex virus in one case, and *Cryptococcus neoformans* in one case. Seventeen of the 35 patients were African Americans, 17 had European ancestors, and 1 patient was born in Haiti. All died in the late 1980s or early 1990s, before HAART was introduced as a treatment for AIDS. Survival time ranged from 2 to 84 months after the diagnosis of AIDS was established. Known opportunistic disease in these 35 AIDS patients were pneumonia from *Pneumocystis carinii* (23/35), mycobacterial disease (15/35), cryptococcosis (5/35), Kaposi sarcoma (5/35), and lymphoma (3/35). An autopsy of the brain was performed in 20 patients. Infection with *C. neoformans* was detected in four cases, with *T. gondii* in three cases. No case of PML was diagnosed.

The 21 control eyes had been enucleated after central retinal vein occlusion, trauma, or end-stage glaucoma of patients without known immunodeficiency. They were formalin fixed and paraffin embedded by standard procedures before two to three sections were cut from each for further experiments.

Two approaches for DNA extraction from paraffin-embedded tissue

Paraffin-embedded sections were studied for DNA of JCV, HIV, and CMV by PCR. Each slide was processed separately for DNA extraction under sterile conditions. Tissue was scraped off the glass with a sterile scalpel and put into a 2-ml Eppendorf tube. The probes were deparaffinized with 1,200 μl of xylol (Fluka, catalog no. 95692), centrifuged at maximum speed for 5 min, and rehydrated with 2 times 1,200 μl of ethanol (J.T. Baker, catalog no. 8006). After evaporation of the ethanol, 100 to 200 μl of digestion buffer was added to each tube. Digestion buffer was composed of 10 mM Tris, 1 mM EDTA (GibcoBRL, catalog no. 15706-021), 1% TWEEN 20, and 0.04% proteinase K (*w*/*v*) at pH 8 (Appligene, catalog no. 130203). After incubation for 48 h at 56°C, proteinase K was inactivated at 100°C for 10 min before the tubes were centrifuged for 3 min at 10,000 rpm and stored at −20°C (method A). In order to optimize the extraction procedure, DNA was purified from the digested and heat-inactivated samples using columns for DNA absorption in method B (QiAmp DNA Mini Kit; Qiagen, catalog no. 51304) following the manufacturers' instruction. Samples were eluted in 50 to 100 ml 10 mM Tris and stored at −20°C until further processed. DNA integrity was controlled by PCR amplification of a 110-bp and a 355-bp fragment from the β-globin gene, using primer pairs BEG-1 and -2 and BEG-3 and -4, respectively (Table 2). This was performed with each sample for verification of the DNA content of each probe. A good sensitivity with low-level background amplification was achieved by a 45-cycle, two-step program starting with a 15-min activation at 95°C of the Hotstar DNA polymerase (Quiagen, catalog no. 203203), 1 min at 55°C for annealing, 1 min at 72°C for elongation, and 1 min at 94°C for denaturation. This was followed by a final 10-min elongation step at 72°C and termination at 4°C.

Table 2 Primers used for amplification of human or viral genes

Primer	*Gene*	*Sequence (5′ → 3′)*	*Length (bp)*
JLP-1	JCV VP1	CTCATGTGGGAGGCTGT(G/T)ACCT	110
JLP-4		ATGAAAGCTGGTGCCCTGCACT	
JLP-15	JCV VP1	ACAGTGTGGCCAGAATTCCACTACC	215
JLP-16		TAAAGCCTCCCCCCCAACAGAAA	
JRR-25	JCV VCR	CATGGATTCCTCCCTATTCAGCA	463
JRR-28		TCACAGAAGCCTTACGTGACAGC	
BEG-1	ß-Globin	ACACAACTGTGTTCACTAGC	110
BEG-2		CAACTTCATCCACGTTCACC	
BEG-3	ß-Globin	ATGGTGCATCTGACTCCT	355
BEG-4		GCCATCACTAAAGGCACCGAGC	
CMV-5	CMV DNA Pol	GAGAGAAGCGCCACATACAG	135
CMV-6		CTACAAGTGCAGCGACGATCCC	
SK-38[a]	HIV Gag	ATAATCCACCTATCCCAGTAGGAGAAAT	115
SK-39		TTTGGTCCTTGTCTTATGTCCAGAATGC	

[a]From the Gene Amplimer HIV-1; Applied Biosystems, part no. N808-0015.

PCR for JCV, CMV, and HIV

Primers specific for JCV, CMV and the ß-globin gene were designed using the Oligo software (Table 2). Proviral DNA from HIV gag gene was amplified with the Gene Amplimer HIV-1 (Applied Biosystems, part no. N808-0015). All reactions were performed with the Hotstar DNA polymerase (Quiagen, catalog no. 203203).

For JCV, each β-globin–positive sample was first analyzed with primers JLP-15 and -16, which amplify a 215-bp fragment in the upstream VP1 gene region. This fragment provides sites that identify all major genotypes and additional subtypes and has been validated as described previously (Agostini *et al*, 1999). Samples negative in the PCR with primers JLP-15 and -16 were further analyzed with primers JLP-1 and -4, which generate a 110-bp fragment inside JLP-15 and -16 primers. In addition, the sequence of the viral control region was obtained using primers JRR-25 and -28, which amplify a 463-bp fragment, including three typing sites on the early side of *ori* (Agostini *et al*, 1997b). For targets within the JCV genome, the PCR program included an initial activation step for the polymerase, followed by a denaturation step at 95°C for 15 min and the 48-cycle three-step PCR program with 30 s at 94°C for denaturation, 1 min at 63°C for annealing, and 1 min at 72°C for elongation. A final 10-min elongation step at 72°C was added before the reaction was stopped at 4°C.

For CMV-specific PCR with primers CMV-5 and -6, the reaction was modified as follows: 15 min activation at 95°C, followed by a 48-cycle three-step program consisting of denaturation for 30 s at 94°C, annealing and elongation for 1 min at 57°C and 72°C, respectively, and a final 5-min extension at 72°C before the reaction was stopped at 4°C. For HIV-specific primers, annealing took place at 60°C and $MgCl_2$ concentration was increased to 2 mM.

Cycle sequencing

Following purification by gel electrophoresis using the Quiagen Gel Extraction Kit (catalog no. 287041), PCR products of the VP1 and regulatory regions were used as template for direct cycle sequencing with the according primers. Primers were end labeled with [^{33}P]-ATP (250 μCi; Amersham Pharmacia Biotech, catalog no. AH9968) and mixed with 2% to 5% of the cleaned template, chain-terminating nucleotides, and a thermostable DNA polymerase (SequiTherm EXCEL II DNA Sequencing Kit; Epicenter Technologies, catalog no. SEM79100). A 5-min activation step at 95°C was followed by 30 cycles of 1 min annealing and elongation at 65°C and 40 s denaturation at 95°C. The products were analyzed on a 6% polyacrylamid gel containing 7 M urea (Gibco Sequencing Mix; Life Technologies, catalog no. 10324-010) as described previously (Agostini *et al*, 1995).

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