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## Identification of Human Immunodeficiency Virus Sequences by Using In Vitro Enzymatic Amplification and Oligomer Cleavage Detection

SHIRLEY KWOK,<sup>1</sup> DAVID H. MACK,<sup>1</sup> KARY B. MULLIS,<sup>2†</sup> BERNARD POIESZ,<sup>3</sup> GARTH EHRLICH,<sup>3</sup>  
DONALD BLAIR,<sup>4</sup> ALVIN FRIEDMAN-KIEN,<sup>5</sup> AND JOHN J. SNINSKY<sup>1\*</sup>

Departments of Diagnostics Research<sup>1</sup> and Human Genetics,<sup>2</sup> Cetus Corporation, Emeryville, California 94608; Division of Hematology/Oncology<sup>3</sup> and Division of Infectious Diseases,<sup>4</sup> Department of Medicine, State University of New York Upstate Medical Center, Syracuse, New York 13210; and Department of Dermatology and Microbiology, New York University Medical Center, New York, New York 10016<sup>5</sup>

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Human immunodeficiency virus (HIV) has been associated with acquired immunodeficiency syndrome and related disorders. Assays to detect antibodies to HIV proteins have been developed and used to screen sera for the identification of individuals who have been exposed to the virus. Although these serological tests have significant sensitivity and specificity for detecting exposure to the virus, they do not provide direct identification of HIV. We report here the application of recently developed nucleic acid amplification and oligonucleotide-based detection procedures for the identification of HIV sequences in established infected cell lines and in cells cultured from infected individuals.

The virus associated with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC), human immunodeficiency virus (HIV), is a member of the *Lentivirinae* (prototype, visna virus) (1, 7, 34). Like visna virus, HIV can establish persistent infection without actively producing virus particles (9). Consequently, immunological assays directed to viral antigens may not detect the virus during some stages of infection. The lengthy prodrome for HIV (4 to 7 years), the need to monitor patients on therapeutic regimens, and the potential transmissibility of HIV from asymptomatic seronegative individuals (8, 13, 14, 27, 28) necessitate the development of a sensitive assay for the viral genome (26). By Southern blot analysis, HIV sequences can be detected in the peripheral blood lymphocytes (PBLs) or tissues (lymph node, liver, kidney, etc.) of only a fraction of AIDS patients (32). Harper et al. (11) using in situ hybridization showed that less than 1 in 10,000 cells of the lymph node of infected individuals contains viral nucleic acid sequences. We report here the identification of HIV sequences in infected cell lines by using an in vitro DNA amplification procedure (K. B. Mullis and F. Faloona, *Methods Enzymol.*, in press) and an oligonucleotide-based detection procedure (25).

The nucleic acid amplification procedure, polymerase chain reaction (PCR), uses two primers of known sequence positioned 10 to 300 base pairs apart that are complementary to the plus and minus strands of target DNA. After denaturation of the target DNA and annealing of the primers, template-directed incorporation of deoxynucleoside triphosphates occurs with the addition of the Klenow fragment of *Escherichia coli* DNA polymerase I. Since the newly synthesized DNA strands may serve as templates themselves, repeated cycles of denaturation, primer annealing, and extension result in an exponential increase of copies of the region flanked by the primers. Saiki et al. (26), using the

procedure, demonstrated a 220,000-fold amplification of a 110-base-pair region of the  $\beta$ -globin gene.

Our plans to apply this amplification procedure to the HIV nucleic acid sequences had to take into consideration the extensive heterogeneity of the viral genome (1, 5, 10, 22, 24, 32, 37). Since sequence alterations can affect primer annealing, only invariant regions of the viral genome were selected for amplification. Highly conserved regions of four HIV isolates were identified by using computer algorithms. The open reading frame encoding the major nucleocapsid protein (*gag*) was particularly conserved in its nucleic acid sequence and was chosen as the region to undergo primer-directed amplification. The selected regions were screened against the DNA sequences of human T cell lymphotropic virus type 1 (HTLV-I) (31) and HTLV-II (33) to ensure that the oligonucleotides would not also prime synthesis from these known human lymphotropic viruses. In addition, characteristics such as primer length, G+C content, and intrastrand complementarity were taken into consideration in primer selection.

Amplified sequences were detected by use of a technique termed oligomer restriction (OR) (25). In OR, an end-labeled oligonucleotide probe hybridizes in solution to a region of the amplified sequence and, in the process, reconstitutes a specific endonuclease cleavage site. Cleavage with the specific endonuclease generates an oligonucleotide of defined size. The requirement of a specific endonuclease cleavage site for OR further defines the region of the viral genome amplified.

Two primer pairs were used to provide corroborative data and to ensure signal generation if sequence alterations occurring in one of the regions targeted for amplification precluded primer or probe binding. The sequences and locations of the oligonucleotides used as primers or OR probes and the resulting diagnostic oligomers after cleavage are given in Table 1.

In preliminary model studies, the  $\beta$ -globin gene was detected in as little as 4 ng of chromosomal DNA (Molt-4 [GM2219C]; Human Genetic Mutant Repository, Camden,

\* Corresponding author.

† Present address: Xytronix, San Diego, CA 92121.

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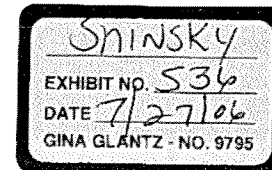


TABLE 1. Sequences of synthetic oligonucleotide primers and probes and their locations in AIDS viral genomes\*

Primer or probe	Sequence	Length (bp) of amplified product	Location of amplified product or probe (nt)
Primer			
SK02	(3') AGTCTGTCCTAGTCTTC (5')	107	900-1006, HTLV-III 448-555, LAV
SK01	(5') CAGGGAGCTAGAACGAT (3')		904-1010, ARV-2 912-1018, LAVA
SK18	(3') CGTAAGACCTGTATT (5')	88	1555-1642, HTLV-III 1104-1191, LAV
SK17	(5') CCAGTAGGAGAAAAT (3')		1565-1652, ARV-2 1567-1654, LAVA
Probe	<i>Bst</i> NI		
SK03	(5') AATCCTGGCCTGTTAGAAAACATCAGAAG (3')		925-952, HTLV-III 474-501, LAV 929-956, ARV-2 937-964, LAVA
	↓		
SK19	(5') ATCCTGGGATTAATAAAAATAGTAAGAATGTATAGCCCTAC (3')		1585-1625, HTLV-III 1134-1174, LAV 1595-1635, ARV-2 1597-1637, LAVA
	↓		
	<i>Bst</i> NI		

\* The primers and probes are within the coding sequence (*gag*) for the nucleocapsid protein. The locations of the amplified products and probes were determined from the available sequence information for HTLV-III (23), lymphadenopathy-associated virus (LAV) (36), and ARV-2 (29), and from an HTLV-III/LAV sequence reported by Muesing et al. (18) (designated LAVA). The primers SK02 and SK18 are complementary to the viral plus strand; SK01 and SK17 are complementary to the viral minus strand. Probes SK03 and SK19 are complementary to the minus strand of the AIDS viral genomes and are used to detect SK01/SK02 and SK17/SK18 amplified products, respectively. The unique *Bst*NI endonuclease site used in the OR procedure is indicated. The arrows indicate the cleavage sites for *Bst*NI in SK03 and SK19. After cleavage with *Bst*NI, SK03 and SK19, if hybridized to complementary sequences, generated a diagnostic labeled pentamer and tetramer, respectively. bp, Base pairs; nt, nucleotides.

N.J.) by PCR and OR (only 2% of the amplified product was analyzed) (Fig. 1, lane 8). The primer pair and probe used for the amplification and detection of  $\beta$ -globin has been described (25, 26). In reconstitution experiments in which Molt-4 was diluted by as much as 250-fold with chromosomal DNA lacking the  $\beta$ -globin gene (GM2064) (35), amplification occurred to nearly an equivalent extent (Fig. 1, lane 9). A recombinant plasmid containing 180 base pairs of the *gag* region of HIV, corresponding to residues 1470 to 1649 of an HIV isolate, AIDS-associated retrovirus (ARV-2) (29), was constructed by using synthetic oligonucleotides (data not shown). The plasmid served as a template to measure the efficiency of amplification with primer oligonucleotides SK17 and SK18. When the recombinant plasmid was spiked at the equivalent of a single-copy gene ( $2.5 \times 10^{-19}$  mol) into 1  $\mu$ g of Molt-4 DNA, the efficiency of amplification, measured by the method described by Saiki et al. (26), was 70%. Satisfied that the DNA amplification and OR procedures allowed detection of specific nucleic acid sequences with greater sensitivity than Southern blot analysis, we embarked on studies using the selected HIV genomic primers.

Initial experiments were performed on DNAs from established cell lines harboring various human lymphotropic viruses. One microgram of the cellular DNA was amplified in a total volume of 100  $\mu$ l for 20 cycles with either SK01/SK02 or SK17/SK18, essentially as described previously (25). For OR analysis, 10  $\mu$ l of the total amplified DNA reaction volume (140  $\mu$ l) was annealed to the [ $^{32}$ P]ATP end-labeled probe (either SK03 or SK19; Table I), digested with *Bst*NI, and one-fourth of this material was analyzed by polyacrylamide gel electrophoresis and autoradiography. Coded samples from seven cell lines infected with HTLV-I (for example, Fig. 2, lanes 7 to 12) and two known to harbor HTLV-II (for example, Fig. 2, lane 6) were repeatedly negative after

PCR-OR analysis. Uninfected cell lines (Molt-4, H9, and HUT 78) were also negative (Fig. 2, lanes 1 to 4). On the other hand, if DNAs from cell lines infected with different isolates of HIV (for example, Fig. 2, lane 5) were analyzed by the same procedures, diagnostic labeled tetra- and penta-oligonucleotides were generated. The data suggest that the PCR-OR procedure can serve to identify HIV sequences and that multiple isolates can be identified. Presumably, both integrated and unintegrated forms of the viral DNA genome may serve as templates for amplification, and as a result, the signal generated in this system should reflect the total cellular viral DNA load.

We next chose to determine whether these procedures

1 2 3 4 5 6 7 8 9 10



FIG. 1. Reconstitution studies using  $\beta$ -globin gene as target sequences for amplification. DNA from Molt-4 was amplified by 20 cycles of PCR with the  $\beta$ -globin primers PC03 and PC04 in the presence or absence of excess DNA from GM2064, as previously described (25, 26) except that the elongation was performed at 25°C. The DNAs used for enzymatic amplification were 996 ng of GM2064 (lane 1), 100 ng of Molt-4 (lane 2), 100 ng of Molt-4 plus 900 ng of GM2064 (lane 3), 60 ng of Molt-4 (lane 4), 60 ng of Molt-4 plus 940 ng of GM2064 (lane 5), 20 ng of Molt-4 (lane 6), 20 ng of Molt-4 plus 980 ng of GM2064 (lane 7), 4 ng of Molt-4 (lane 8), 4 ng of Molt-4 plus 996 ng of GM2064 (lane 9), and an OR control (100 ng of Molt-4) (lane 10).

1 2 3 4 5 6 7 8 9 10 11 12

FIG. 2. Determination of presence of AIDS viral sequences in cell lines by using PCR-OR. Propagation of virus-infected cell lines (19) and extraction of DNAs (17) were performed as described. One microgram of the indicated cell line DNA was amplified for 20 cycles with SK01 and SK02 and analyzed by OR with probe SK19 and *Bst*NI (25, 26). Lanes: 1, Molt-4; 2, H9 (21); 3, HUT 78 (6); 4, uninfected T cells; 5, HIV (K. Wells, G. Ehrlich, and B. Poiesz, manuscript in preparation)-infected HUT 78; 6, MoT (HTLV-II) (15); 7, MT2 (HTLV-I) (38); 8, HUT 102B2 (HTLV-I) (20); 9, H516 (HTLV-I); 10, UMC CTL-1B (HTLV-I); 11, UMC CTL-2 (HTLV-I); 12, UMC CTL-3 (HTLV-I).

could be used to identify viral nucleic acids in cultured cells infected with specimens from seropositive AIDS and ARC patients. The specimens included PBLs, semen mononuclear cells, and semen supernatants. Our success was nearly equivalent with the three different specimen sources. Of 19 DNA samples from cultures demonstrating reverse transcriptase (RT) activity, 17 generated a diagnostic oligomer with either or both primer pairs with the PCR-OR procedure (for example, Fig. 3A, lanes 1 and 5 to 7) (Table 2). In comparison, only 11 of 18 samples tested were positive by Southern blot analysis with a nick-translated probe, the 6.5-kilobase *Hind*III fragment of pBenn6 (gift of M. Martin). These results suggest that the amplification-based detection procedure is more sensitive than direct blotting. The experience of numerous investigators suggests that nucleic acid sequences present at less than one copy in 10 cells cannot be unambiguously identified (32) with the Southern blot procedure. The observation that some samples scored positive with only one of the two primer pairs suggests sequence alterations may have occurred within the region complementary to the primer pair or probe or both. To address this possibility, dot blotting of PCR-amplified DNA was performed with the labeled oligonucleotide probe. Hybridization conditions were chosen such that a small number of sequence alterations would not prevent oligonucleotide binding. Whereas samples that were positive by OR hybridized to the radioactive probe, we were unable to detect hybridization of the probe to samples that were negative by OR (data not shown). We conclude that sufficient sequence alterations have occurred in the primer-binding region(s) of the viral genome to prevent amplification of DNA from these cell lines.

In addition, the intensity of the labeled diagnostic oligonucleotide was not proportional to the level of RT activity measured. This observation was not unexpected since visna virus, a related lentivirus, is known to accumulate linear DNA viral genomes even though they are transcriptionally dormant (2). In addition, since a variable number of the viral DNA genomes present may be transcriptionally active, a correlation of viral DNA copy number and virus particles (as measured by RT activity) would not necessarily be expected.

Of concern were two DNA samples that, although positive for RT activity, were negative by the PCR-OR procedure (for example, Fig. 3B, lane 4). However, the RT activity of

these samples (about 2.0 pmol) was near the designated threshold of the assay (0.5 pmol of [<sup>3</sup>H]GMP above background). Given the variability observed within and between RT assays, samples showing minimal incorporation of labeled deoxynucleoside triphosphates should be evaluated cautiously. Successful amplification with the previously described  $\beta$ -globin primer pair (26) suggested that the inability to identify these RT-positive samples was not due to non-specific inhibition of amplification or OR. Additional attempts using 25 cycles (theoretically allowing a  $[5.77 \times 10^2]$ fold amplification at 70% efficiency) and 10 times more DNA or other primer pairs were unsuccessful (data not shown). We conclude that the inability to identify some RT-positive cell lines reflects (i) an exceptionally low copy number of viral DNA genomes, (ii) sequence alterations in the region complementary to the primers and probes, (iii) some combination thereof, or (iv) artifactual RT activity. We are continuing to examine the RT-positive, PCR-OR-negative DNA samples to determine the reason for our inability to detect these samples.

Of particular note was the ability of the PCR-OR procedure to identify HIV sequences in 9 to 41 cell lines that were negative for RT activity (Fig. 3C, lane 2). Of the 41 cell line DNAs, 28 were analyzed by Southern blotting, and 3 were identified as positive (Table 2). One of the three samples gave a low-molecular-weight smear and was negative by PCR-OR. Since cell lines not exhibiting RT activity are typically discarded as virus negative, the PCR-OR procedure applied to all cultures may be informative. Recent studies indicate that such cells may be induced to produce virus after treatment with a halogenated pyrimidine (5'-iodo-2'-

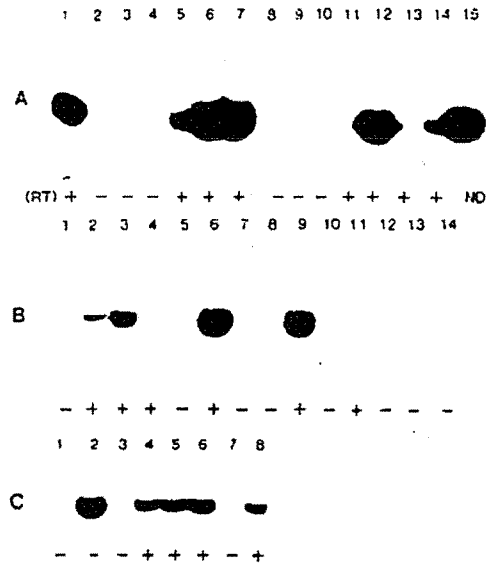


FIG. 3. Representative PCR-OR analyses of cells propagated from individuals with AIDS and ARC. DNA samples (1  $\mu$ g) were amplified for 20 cycles with primer pair SK01/SK02 (A and B) or SK17/SK18 (C) and detected by cleavage of SK03 or SK19 with *Bst*NI. The plus or minus sign below each lane indicates the presence or absence, respectively, of RT activity in culture supernatants. RT activity was measured as previously described (19). The sample in which RT activity was not determined (ND) is indicated.

TABLE 2. PCR-OR and Southern blot analyses of cell lines propagated from AIDS and ARC patients\*

Primer pair(s) for which cell line OR positive	No. of cell lines with RT activity:					
	Positive		Negative		Not determined	
	PCR-OR (19)	Southern blot (18)	PCR-OR (41)	Southern blot(28)	PCR-OR (7)	Southern blot (5)
SK01-SK02 and SK17-SK18	11	9/10	1	ND <sup>b</sup>	1	ND
SK01-SK02 only	2	1/2	1	0/1	0	ND
SK17-SK18 only	4	1/4	7	1/2	1	ND
Neither	2	0/2	32	2/25	5	0/5

\* The cell lines are grouped according to the RT activity in the culture supernatants. The numbers in parentheses below the procedure used for analysis indicate the total number of DNA samples examined. The fractions shown for Southern blot analysis indicate the number positive of the number tested. For Southern blot analysis, 25 µg of DNA was used. The blots were autoradiographed for 48 to 72 h. The probe, pBenn6, was labeled to a specific activity of  $5 \times 10^8$  to  $1.2 \times 10^9$  cpm/µg with [<sup>32</sup>P]dCTP. The results are subdivided into those positive by PCR-OR analysis for both primer pairs independently or only one of the two primer pairs or negative for both primer pairs independently. For PCR-OR analysis, samples of DNA (1 µg) from the cultured cells were amplified for 20 cycles with SK01/SK02 with SK03 as the labeled oligonucleotide for OR analysis, and with SK17/SK18, with SK19 as the labeled oligonucleotide for OR analysis.

<sup>b</sup> ND, Not determined.

deoxyuridine) (3). The simplicity and rapidity with which PCR-OR analysis can be performed suggests it as a viable supplemental procedure to induction of nonproducing persistently infected cell lines. Of seven DNA samples in which RT activity was not determined, two were positive by PCR-OR (Table 2).

In summary, we demonstrated that the *in vitro* enzymatic amplification and OR detection procedures can identify HIV sequences in both established cell lines harboring HIV (but not those harboring HTLV-I and -II) and in cells cultured from PBLs, semen mononuclear cells, and semen supernatants of AIDS or ARC patients. Viral nucleic acid sequences were detected in cell lines that were devoid of detectable RT activity. In addition, these procedures identified HIV sequences in infected cells that were negative by Southern blot analysis. Experiments in progress include examination of DNA of PBLs taken directly from patients at risk for or with AIDS and continued exploration of alternate primer pairs for PCR. Preliminary results demonstrate that the PCR-OR procedure can be used successfully to detect viral sequences in DNA isolated from PBLs. A further focus of our studies is the continued investigation of the RT-positive, PCR-OR-negative samples. It will be of interest to determine whether the virus responsible for disease in some patients is considerably different in nucleic acid sequence than viral genomes sequenced to date.

Experiments similar to those reported here may prove fruitful, in general, for the detection of other viruses and specifically for the detection of latent viruses. Perhaps of particular note are cytomegalovirus (30), hepatitis B virus (16), and Epstein-Barr virus (4), all suggested to reside at some stage within lymphocytes and possibly to play a role in AIDS (12).

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