Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 311-317

RECOMBINATION BETWEEN HUMAN IMMUNODEFICIENCY VIRUSES (HIV) TYPE 1 AND 2 RESULTS IN GENERATION OF DEFECTIVE HYBRID VIRUSES

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Received April 8, 1993

SUMMARY Dual infection by HIV-1 and HIV-2 is already documented. To test if this could lead to recombination and generation of altered viruses, recombination between HIV-1 and HIV-2 DNAs was studied. Release of recombinant viruses was detected following cotransfection of human rhabdomyosarcoma cells with truncated versions of these proviral DNAs. Linearization of plasmid DNAs was required for virus production. Analysis of viral particles by hybridization revealed the presence of viral RNA. However viral replication was not evident. A computer search of the overlap region between the substrate DNAs revealed 66% homology despite an overall genomic sequence homology of only 35%. These data suggest possible generation of replication-deficient hybrid viruses as a result of recombination between HIV-1 and -2.

The two prototype human immunodeficiency viruses HIV-1 and HIV-2 exhibit differences in their geographic distribution. HIV-1 was primarily isolated from AIDS patients in central Africa, the United States, and western Europe (1-3), while HIV-2 is generally limited to west African countries, with sporadic occurrences in central Africa, Europe and US (4-6). Biologically, different isolates of HIV-2 exhibit properties different from that of HIV-1, such as attenuated cytopathicity and reduced capacity for cell-free transmission (7), lack of CD4 receptor down-regulation (8) and decreased virulence (9). At the genome level HIV-2 differs from HIV-1 in having a long terminal repeat (LTR) longer by about 200 base pairs (10), a truncated transmembrane domain of the envelope glycoprotein and presence of a 16 kD protein termed VPX (11,12).

HIV infection is also accompanied by a variety of viral and/or other opportunistic pathogens in more than 50% of the infected individuals. Dual infections of the same individuals with HIV-1 and HTLV-I or HTLV-II (13) and, more important, with HIV-1 and HIV-2, have been reported by several groups (14-17). Proviral DNAs of HIV-1 and HIV-2 have also been simultaneously detected from patients, which leads to a scenario of possible recombination between the viral DNAs. In addition, the diploid nature of virion RNA in viral particle provides an opportunity to package HIV-1 and HIV-2 RNA in the same particle during virion morphogenesis. Recombination between virion RNAs during reverse transcription is already documented (18). These observations prompted us to verify recombination between HIV-1 and HIV-2.

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0006-291X/93 $4.00

In order to test for recombination at DNA level between HIV-1 and HIV-2 (which exhibit only partial nucleic acid homology), we used truncated clones of HIV-1 and HIV-2 as substrate DNA for transfection into human cells. Since recombination between exogenous DNA sequences have been extensively studied utilizing transfection of DNA into cultured cells (19-21), this approach was chosen for our studies. The results reported here indeed provide evidence that recombination between HIV-1 and -2 proviral DNA is possible.

MATERIALS AND METHODS

Cells and plasmids Monolayer cultures of human RD cells and suspension cultures of SupT1, Hut 78 and CEMX174 cells were maintained in DMEM and RPMI 1640 media, respectively, both supplemented by 10% FBS, 100 u/ml penicillin and 100 u/ml streptomycin. Phytohemagglutinin (PHA) -stimulated peripheral blood lymphocytes (PBL) were prepared as described (22). Egp and KXba plasmids represent 5' and 3' partial clones of HIV-2 in bluescript plasmid (kindly provided by G. Franchini). The proviral HIV DNAs pZ6Neo (23, from which partial clones were derived), pARV (24) and pXba (25) have already been described. In each truncated proviral clone, either the 5' or the 3' LTR was retained.

Transfection Subconfluent RD cells were trypsinized and 2 x 10^6 cells/10 cm petri dish were plated 20 hours prior to transfection. $Ca_3(PO_4)_2$-DNA complexes were precipitated as described (26) using (i) 10 or 20 μg of plasmid DNA or (ii) 10 μg of each of the two plasmids in the case of co-transfection. The cells were exposed to the precipitate for 8 hours followed by a 90 sec. glycerol shock (27).

Infectivity of recombinant viruses Media from the plates were collected on days 3 and 5 post-transfection, clarified by centrifugation at 16000g x 10' at 4°C and divided into 2 parts. One aliquot was used for the assays while another was used to infect SupT1 or CEMX174 cells. Infectivity of the recombinant viruses was tested by the following methods: (a) mixing an aliquot of the supernatant with 3 x 10^6 SupT1 or CEMX174 cells overnight followed by washing the cells three times in PBS and resuspending in fresh RPMI medium. Supernatant of these cells were collected on days 2, 4, 6, 8 and 10 for p24 antigen assay or reverse transcriptase (RT) assay; (b) 3 x 10^6 SupT1 or CEMX174 cells were cocultivated with transfected RD cells for 2 days prior to 3 washes with phosphate buffered saline and resuspension in fresh RPMI medium. Supernatants of day 2, 4, 6, 8 and 10 after resuspension in RPMI were assayed for p24 antigen. Viral lysates used for p24 and RT assays were prepared by precipitating 1.2 ml of clarified supernatant with half volume of 30% (w/v) polyethylene glycol in the presence of 0.5 M NaCl at 4°C overnight. The precipitate was pelleted in the microfuge and lysed in 80 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.25 M KCl, and 0.25% Triton X-100).

HIV antigen and RT assays The p24 antigen assay was performed according to the manufacturer's (Coulter, Inc.) instructions. The RT assay was practically similar to a published method (2).

PCR analysis Primers specific for HIV-1 CCACCTATCCCAGTAGGAGAAAT and CCACCTATCCCAGTAGGAGAAAT (and GGTCCTTGTCTTATGCCAGAATGC), HIV-2 (CAGCTGCAGGGACTTACTAT and AAGCAGAGAGTCTCAGTGTC) and cross-over primers derived from both (CCCTTTCGTTCATAACATATCC) were selected from appropriate conserved sequences and synthesized in-house. PCR was performed with cellular extract containing both high molecular weight and Hirt DNAs using the reagents and guidelines from the manufacturer (Perkin-Elmer). The parameters used were 1 min, 1 min, and 2 min at 95°C, 55°C and 72°C for denaturation, annealing and polymerization respectively, with 30 cycle reactions.

Southern hybridization 15 μl out of 50 μl PCR reaction mix was resolved on a 1.5% agarose gel, transfered to nylon membrane and hybridized to ^{32}P-labeled 5.8 kb, HIV-2 specific probe followed by autoradiography at -70°C, according to guidelines from S & S (Keene, NH).

Sequence analysis The overlap region between HIV-1 and HIV-2 clones were compared for nucleotide sequence homology using the software *bestfit* on a Macintosh II computer.

RESULTS

Deletion mutants as substrates for recombination: In order to test for recombination, mutants of HIV-1 (HIVZ6) possessing deletions of *gag* and *pol* genes to varying extents, designated ZBam, ZEco, ZSca, ZApa, and ZSph were used as a source of 5' viral DNA sequences in transfection experiments (Fig. 1A). The 3' sequence was provided by a partial clone of HIV-2, designated KXba (Fig. 1B). Likewise, two mutants that provide the templates reciprocal to the first set (designated Egp-Sac 5' and Xba-Nar 3'), were also used. Successful recombination between truncated proviral DNAs was monitored by quantitating the viral particles released from the transfected RD cells by p24 antigen assay.

Optimal condition for recombination: When circular plasmid DNAs were used as substrates for recombination, only homologous, not heterologous, clones gave positive p24 values (Table 1). In contrast, when plasmids ZBam and KXba were both linearized prior to transfection, significant p24 values were obtained. Since linearization of DNA facilitates annealing of homologous sequences, this observation indicates possible recombination between ZBam and KXba DNAs resulting in the synthesis of viral particles through generation of genomic and subgenomic RNAs.

Requirement for recombination: For further confirmation of this phenomenon, truncated versions of HIVZ6 proviral DNA representing different extents of deletion were transfected singly and also in

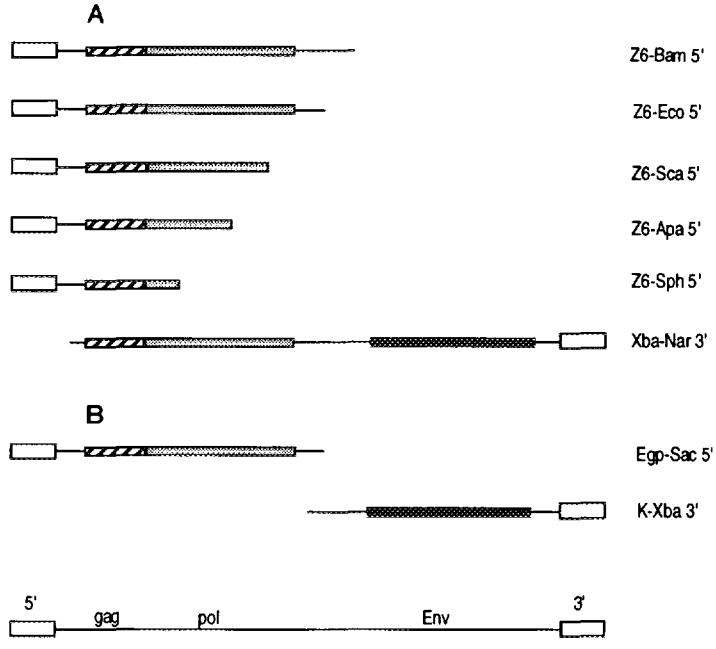

FIG. 1. Restriction map of the truncated proviral clones used in transfection experiments. The name of the clone is hyphenated with the restriction enzyme cleavage site used for creating the deletion. LTRs are shown as open boxes. The primed numbers refer to the corresponding part of the viral genome. (A) Fragments derived from HIV-1 sequences. (B) Fragments derived from HIV-2 sequences.

TABLE 1. OPTIMAL CONDITIONS FOR HOMOLOGOUS AND HETEROLOGOUS RECOMBINATION

Substrate DNA		*day post*	*Plasmid DNA*	
5'	*3'*	*transfection*	*Circular*	*Linear*
Homologous				
Z6-Bam	HX-Sal3	3	.88	2.0
Egp-Sac	K-Xba	3	.58	1.68
Heterologous				
Z6-Bam	K-Xba	3	.01	2.44
		5	.1	1.9

Linear refers to plasmid DNAs that were digested with restriction enzymes XbaI and BamHI, extracted with phenol and ethanol precipitated before transfection. Numbers indicate ^{450}A values (mean of 2 experiments). Assay controls: +ve: 1.21; cut off: 0.1.

combination with KXba into RD cells. All mutants of HIV-1 and HIV-2 gave negative results for p24 antigen when transfected singly. So was the case with the cotransfection of ZSca, ZApa, or ZSph individually with KXba (Table 2), as expected, because these combinations lacked any physical overlap whatsoever. However, combinations of ZBam and KXba as well as ZEco and KXba gave distinctly positive p24 antigen values (Table 2), comparable to that of the provirus pARV, which was a positive control. Even in reciprocal transfection experiments where the 5' fragment was provided by HIV-2 and

TABLE 2. RECOMBINATION BETWEEN TRUNCATED PROVIRAL DNA FRAGMENTS

		P24 antigen (^{450}A units) post transfection	
5' viral DNA	*3' viral DNA*	*Day 3*	*Day 5*
Z6-Bam	–	0.05[a]	0.05
Z6-Eco	–	0.13	0.11
Z6-Sca	–	0.07	0.03
Z6-Apa	–	0.05	0.05
Z6-Sph	–	0.02	0.02
–	K-Xba	0.03[a]	0.05
Z6-Bam	K-Xba	2.85[a]	2.26
Z6-Eco	K-Xba	2.47	2.45
Z6-Sca	K-Xba	0.07	0.05
Z6-Apa	K-Xba	0.05	0.06
Z6-Sph	K-Xba	0.02	0.02
Egp-sac	–	0.1	NT
–	Xba-Nar	0.1	NT
Egp-sac	Xba-Nar	0.8	NT
puc (-ve control)		0.09	0.03
ARV (+ve control)		2.62	2.38
pBS (-ve control)		0.07	NT
pNL4 (+ve control)		3.0	NT

RD cells were cotransfected with 10 μg of each plasmid DNA. Viral pellets isolated on days 3 and 5 were lysed in solubilization buffer and 15 μl of the lysate was used for the assay. Numbers indicate ^{450}A values (mean of 3 or 4 experiments). Assay controls: + ve: 1.2; cut off: 0.12. [a]Mean of 6 experiments. NT, not tested.

the 3' fragment by HIV-1 (Fig. 1), only cotransfections gave positive p24 values. Results from this set of experiments strongly suggest recombination between two viral DNAs with an overlap. Analysis of viral particles spotted on to a nitrocellulose filter revealed the presence of both HIV-1 and HIV-2 specific sequences upon hybridization with respective probes (data not shown).

DNA sequence homology: To test for possible nucleic acid (NA) homology in the overlap region between HIV-1 and HIV-2, a computer search was performed using the software *bestfit* . This search revealed 66% NA homology in the overlap region, including several discrete segments of 9-13 consecutive bases (Fig. 2). Therefore, the input plasmid DNAs from HIV-1 and HIV-2 could have served as potential substrates for homologous recombination within these regions.

Absence of viral replication: The resulting hybrid viruses from such recombination were tested for the biological properties documented for HIV-1 and HIV-2. The hybrid viruses were transmitted to PHA-stimulated PBLs and also established target cell lines such as SupT1, Hut 78 and CEMX174. Viral replication in these cells was followed by monitoring RT and viral p24 antigen assays. Unlike the virus derived from HIV-1 and HIV-2 proviral clones, the hybrid virus showed lack of replication when transmitted as a cell-free virus (data not shown). Infectivity was also tested as a cell-associated virus by co-cultivating the target cells with the transfected RD cells. The results clearly showed that the

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HIV-1   AGGAGTAGTAGAATCTATGAATAAAGAATTGAAGAAAATTATAGGACAGG
HIV-2   AGGAGTAGTAGAAGCAATGAATCACCATCTAAAAAACCAAATAAGTAGAA
HIV-1   TAAGAGATCAAGCTGAGCATCTTAAGACAGCTGTACAAATGGCAGTATTC
HIV-2   TCAGAGAACAGGCAAATACAATAGAAACAATAGTACTAATGGCA..ATTC
HIV-1   AT..CCACAATTTTAAAAGAAAAGGGGGGATTGGGGGATACAGTGCAGGG
HIV-2   ATTGCATGAATTTTAAAAGAAGGGGGGGAATAGGGGATATGACTCCATCA
HIV-1   GAGAGAATAATAGACATAATAGCAACAGACATACAAACTAAAGAATTACA
HIV-2   GAAAGATTAATCAATATGATCACCACAGAACAAGAGATACAATTCCTCCA
HIV-1   AAAACAAATCACAAAAATTCAAAATTTTCGGGTTTATTACAGGGACAGCA
HIV-2   AGCCAAAAATTCAAAATTAAAAGATTTTCGGGTCTATTTCAGAGAAGGCA
HIV-1   GAGATCCAATTTGGAAAGGACCAGCAAAGCTCCTCTGGAAAGGTGAAGGG
HIV-2   GAGATCAGTTGTGGAAAGGACCTGGGGAACTACTGTGGAAAGGAGAAGGA
HIV-1   GCAGTAGTAATACAAGACAATAGTGACATAAAGGTAGTACCAAGAAGAAA
HIV-2   GCAGTCCTAGTCAAGGTAGGAACAGACATAAAAATAATACCAAGAAGGAA
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FIG. 2. Nucleotide sequence homology in the overlap region between HIV-1 and HIV-2 clones. Top and bottom rows represent HIV-1Z6 and HIV-2 sequences, respectively. Only a part of the overlap is shown for the sake of brevity.

hybrid viruses were replication-defective. PCR analysis of total cellular DNA failed to show viral sequences, thus confirming the lack of viral replication (data not shown).

DISCUSSION

Retroviruses, in general, have been shown to accumulate variation in the viral genomes because of an unique, yet error-prone enzyme reverse transcriptase. In addition, the mode of retroviral replication, specifically the processes associated with reverse transcription, also promote genetic recombination between viral genomes which range from 25-40% (18). The presence of multiple viral DNA copies in the infected cells presents a scenario in which recombination between DNAs with homology cannot be ruled out. The study presented here was prompted by the observation of HIV-1 and -2 co-infections in individuals. This led us to explore the curious possibility of the generation of hybrid viruses between HIV-1 and -2. Based on our earlier studies on recombination between HIV-1 DNAs, we have analyzed recombination between HIV-1 and -2 proviral DNAs using truncated viral DNA as substrate.

Our experimental evidence that only cotransfectants yield positive p24 antigen values is a reasonable indicator of recombination. That it additionally requires linear DNA augments this reasoning, since linearization facilitates annealing of homologous sequences by making even short stretches of DNA more accessible for cross-over. Requirement of the presence of a physical overlap between the input DNAs strengthens this arguement. It is further supported by the presence of homologous regions including several segments of 9-13 consecutive bases in this overlap. Similar stretches of homology have been shown to be adequate for recombination (22). A 66% homology in this region is significant since the overall homology between the viral genomes is only about 35%. The release of viral particles from viral DNA transfected cells was used as an index of successful recombination between HIV-1 and HIV-2 DNAs. The viral particles were not merely empty capsids as was evident from the positive hybridization observed with HIV-1 and HIV-2 DNA probes. The inability to elicit either p24 or RT activities or syncytia in established HIV-susceptible cells and cell lines showed that the viral particles released were replication defective. PCR-Southern analysis of total DNA of the cells infected with hybrid viruses showed lack of viral DNA suggesting that the defect may be due to events prior to or during reverse transcription of virion RNA to DNA. The evidence presented here strongly suggests generation of replication-defective hybrid viruses, between viruses as divergent as HIV-1 and HIV-2 due to a high degree of nucleotide homology in defined segments.

ACKNOWLEDGMENTS

This work was supported in part by grant AI 29306 from NIH to A.S. We thank Dr. E. Premkumar Reddy, Fels Research Institute, Philadelphia, PA for his support, Dr. G. Franchini of the National Cancer Institute, Bethesda, MD for providing the Egp and KXba plasmids and the Wistar Institute, Philadelphia, PA for providing the facilities.

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