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Generation of hybrid human immunodeficiency virus by homologous recombination

(AIDS/genetic diversity)

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ABSTRACT **Human immunodeficiency virus (HIV) type 1, isolated from diverse sources, exhibits genomic diversity. The mechanisms by which the genomic diversity takes place in individuals exposed to multiple virus isolates is yet to be elucidated. Genetic variation, in general, might result from mutagenic events such as point mutations, rearrangements (insertions and deletions), and recombination. In an attempt to evaluate the process of genetic diversity, we designed experiments to analyze recombination between HIV DNAs by using DNA transfection in cell cultures. Here we report the successful recombination between truncated HIV proviral DNAs with an overlap homology of 53 base pairs that leads to the formation of viable hybrid virus. Recombination was also seen between exogenous DNA introduced into cells and homologous HIV sequences resident in the cells. These results indicate that recombination among various HIV isolates may play a significant role in the generation of genetic diversity of HIV. Further, the method used here enables the construction of hybrid HIV genomes to identify the viral determinants responsible for tropism, replication, and cytopathic effects.**

Human immunodeficiency viruses (HIV) type 1 and 2 are the causative agents of AIDS (1–5). HIV-1 has been isolated from a number of tissues, including peripheral blood lymphocytes (PBL), cerebrospinal fluid, brain tissue, and macrophages from virus-infected individuals (6–12). HIV-1 isolates from individuals with different disease spectra were shown to have differential ability for replication in various cell lines (13, 14). Thus, HIV-1, isolated from macrophages and cerebrospinal fluid, has shown preferential replication in macrophages and glial cells, respectively (10, 11).

Molecular cloning and characterization of several HIV isolates revealed that nucleotide and predicted amino acid sequences vary to 18% and 33.1%, respectively, in the envelope gene among different isolates. The extent of variation in other genes of HIV ranges between 1 and 8% (15–18). The ability of HIV to undergo changes *in vivo* was further shown in viruses isolated from infected individuals at different intervals and also in viruses isolated from blood donor and recipients (18, 19). The events responsible for genetic variation between different viruses are point mutations, rearrangements, and recombination (16).

The HIV-1-infected cells harbor a large amount of unintegrated viral DNA in addition to the proviral-integrated sequences. Further, accumulating evidence from the literature indicates that multiple viral isolates with different primary nucleotide sequence (18–20) and possibly different biologic properties reside in the same target cell. These observations provide a good scenario for recombination to occur between HIV DNAs to generate viruses with genetically diverse sequences. Due to nonavailability of well-characterized conditional HIV mutants, it has been difficult to study the recombination between HIVs *in vivo* or in cell cultures.

We have used HIV proviral DNAs to analyze the recombination mechanisms. Because recombination of exogenous DNAs shares many features with recombination between endogenous chromosomal segments, we introduced pairs of noninfectious HIV proviral DNAs by DNA transfection and followed their fate in cultured cells. DNA transfection in somatic cells has been effectively used by a number of groups to analyze recombination (21–34). In this study, the recombination method was successfully used to generate hybrid HIV from well-characterized HIV proviral clones.

MATERIALS AND METHODS

Cells. Human rhabdomyosarcoma (RD) and HeLa cells (obtained from the American Type Culture Collection) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and L-glutamine (540 μg/ml) at 37°C with 5% CO_2. HUT 78 cells and phytohemagglutinin (10 μg/ml)-stimulated PBL were maintained as suspension cultures in RPMI 1640 medium containing T-cell growth factor (10%).

Plasmids. HIV DNA plasmids, designated pCDC451, pARV, pZ6Neo, and pXba were derived from cells infected with HIV_{CDC451} (35), HIV_{SF-2} (36), HIV_{Zr6} (37), and $HIV_{HTLV-III}$ (38), respectively. Construction of HIV long terminal repeat (LTR)-chloramphenicol acetyltransferase (CAT) plasmid (pLTR-CAT) and HIV transactivator gene-containing plasmid (pTAT) has already been described (39). To completely rule out the presence of residual homologous DNA fragments, recombinant plasmid constructs containing different regions of viral DNA were prepared by taking advantage of the unique restriction sites present in the viral DNA and also in the vector sequences (Fig. 3). In all cases, 5′ or 3′ LTR was retained.

Transfection. HeLa or RD cell cultures were split 24 hr before transfection, and the growth medium was replaced 1–2 hr before the addition of calcium phosphate-precipitated DNAs that were formed as described (40). Cells (1×10^6) were exposed to the precipitate for 8 hr followed by 90 sec of glycerol shock (41).

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Abbreviations: HIV, human immunodeficiency virus; PBL, peripheral blood lymphocytes; RD, rhabdomyosarcoma; LTR, long terminal repeat; RT, reverse transcriptase; CAT, chloramphenicol acetyltransferase.

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CAT Assay. CAT assays were performed by incubating cellular extracts with [^{14}C]chloramphenicol (New England Nuclear) and acetyl-CoA in a manner similar to that described (42).

HIV Antigen and Reverse Transcriptase (RT) Assays. The HIV antigen ELISA assay (Abbott) was performed according to the manufacturer's guidelines (43). The RT assay procedure was essentially similar to that described (2).

Southern Hybridization. Recombinant viruses were propagated in phytohemagglutinin-stimulated PBL as described (2). High-molecular-weight DNA was extracted 7 days after infection. DNA (10 μg) was digested with *Sac* I and electrophoresed on 0.8% agarose gel. The DNA fragments were transferred to nitrocellulose filters and hybridized to nick-translated full-length HIV probe (37, 44).

RESULTS

Synthesis and Assembly of HIV in RD Cells. To study the recombination between truncated HIV proviral DNA, we initially analyzed the various parameters involved with complete proviral DNA. It was reported earlier by us (45) and other investigators (36, 46) that HIV proviral DNA can be transfected into adherent cells including NIH 3T3 cells, RD cells, HeLa cells, and SW480 colon carcinoma cells. RD cell line, in our laboratory, gave the best results in terms of virus production after transfection with HIV_{Zr6} proviral DNA (45). RD cells were transfected with various concentrations of three different HIV proviral DNAs: pZ6Neo (37) derived from HIV_{Zr6}, pARV (36) derived from HIV_{SF2}, and pXba (38) derived from $HIV_{HTLV-III}$. Production of viral particles was measured directly by RT assay (2). Transfection of HIV proviral DNA pZ6Neo resulted in maximum virus production, followed by pARV (Fig. 1). RD cells showed maximum virus production at day 3 after transfection and in a dose-dependent manner (data not shown). The pXba clone was inactive in virus production by RT assay, although this clone was able to activate LTR-directed CAT expression in transient expression assays similar to other proviral DNAs (Fig. 2).

Homologous Recombination Between Truncated HIV Proviral DNAs. Recombinant plasmid constructs containing different regions of viral DNA were prepared from various proviral DNAs by standard recombinant DNA techniques (Fig. 3). RD cells were transfected with 10 μg of each of these recombinant plasmid DNAs. Release of viruses in the medium was monitored by RT assay and antigen assay. All truncated HIV viral DNAs were inactive in virus production by both assays and therefore offered ideal substrates for recombination assays.

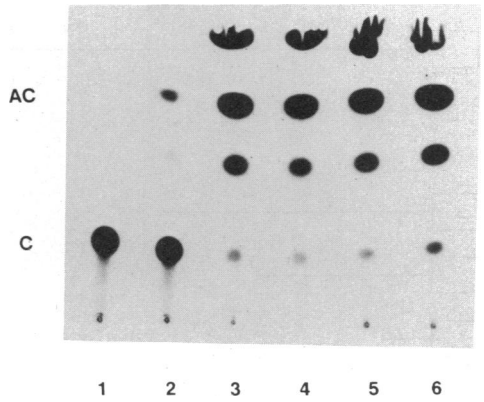

FIG. 2. Activation of HIV LTR-directed CAT expression by HIV proviral DNA. HeLa cell cultures were split 24 hr before transfection. Cells (1×10^6) were transfected with 10 μg of pLTR-CAT or were cotransfected with 10 μg of pLTR-CAT and 2 μg of pTAT. For HIV proviral DNA, 10 μg was used for cotransfection experiments. Cells were harvested 48 hr after transfection. CAT assays were done by incubating cellular extracts with [^{14}C]chloramphenicol and acetyl-CoA as described (42). C, chloramphenicol; AC, acetylated forms of chloramphenicol. Columns: 1, untransfected cells; 2, pLTR-CAT alone; 3, pLTR-CAT and pTAT; 4, pLTR-CAT and pZ6Neo; 5, pLTR-CAT and pARV; and 6, pLTR-CAT and pXba.

Previous studies from our laboratory reported the optimal conditions for efficient recombination between HIV DNAs (48, 49). The optimal conditions of DNA exposure (8 hr) and concentration (10 μg) were used for all transfections. The results observed using cotransfection of different plasmids are presented in Table 1. The results indicate that only the plasmids with overlapping sequences show viral antigen in the medium. Interestingly, plasmids with 5′ ends derived from pXba clone did not show viral antigen in cotransfection experiments. This indicates that the defect in pXba clone may be localized in the 5′ end of the genome.

Recombination Between Exogenous and Homologous Chromosomal HIV Sequences. RD cell lines were generated by cotransfecting the truncated HIV DNA (ARV-Sph 3′) and neomycin (pSV2Neo) marker gene. Cells, growing in the presence of G418 (400 μg/ml), were cloned and tested for the presence of HIV sequences by Southern hybridization and transfection with Z6-Bam 5′ DNA. Recombination between DNA introduced into cells and homologous chromosomal sequences released HIV in the medium (Table 2).

Infectivity of the Recombinant Viruses. Cotransfection of truncated HIV plasmids in RD cells showed viral particles in the medium. Production of virus particles could result either from complementation of viral proteins directed by plasmids used for transfection or from recombination between DNAs,

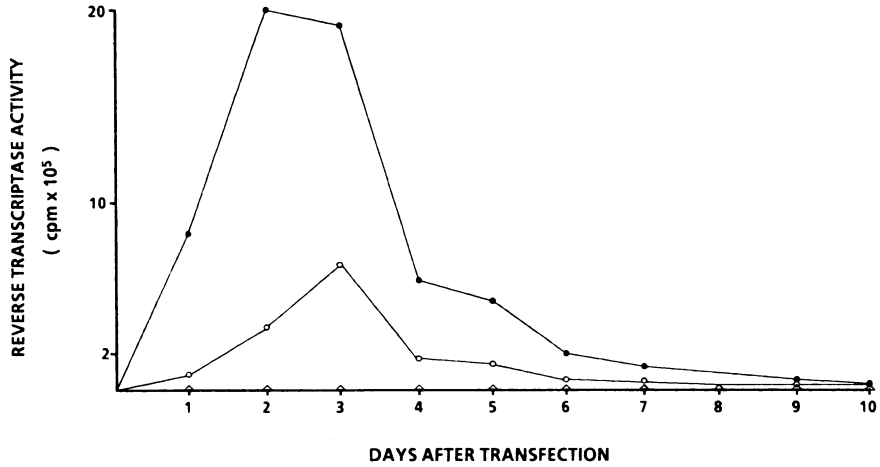

FIG. 1. Production of virus in RD cells transfected with HIV proviral DNA. Kinetics of virus production in RD cells transfected with different HIV proviral DNA. HIV proviral DNA plasmids, designated pARV, pZ6Neo, and pXba, were derived from cells infected with HIV_{SF-2} (36), HIV_{Zr6} (37), and $HIV_{HTLV\ III}$ (38), respectively. The restriction enzyme analyses of pXba clone show similarity to those of other HIV proviral DNAs cloned from H9 cells infected with isolate subtype HTLV-III. Ten micrograms of proviral DNA was used for each transfection, and virus production was monitored for 10 days as described in *Materials and Methods*. ●, pZ6Neo; ○, pARV; and △, pXba.

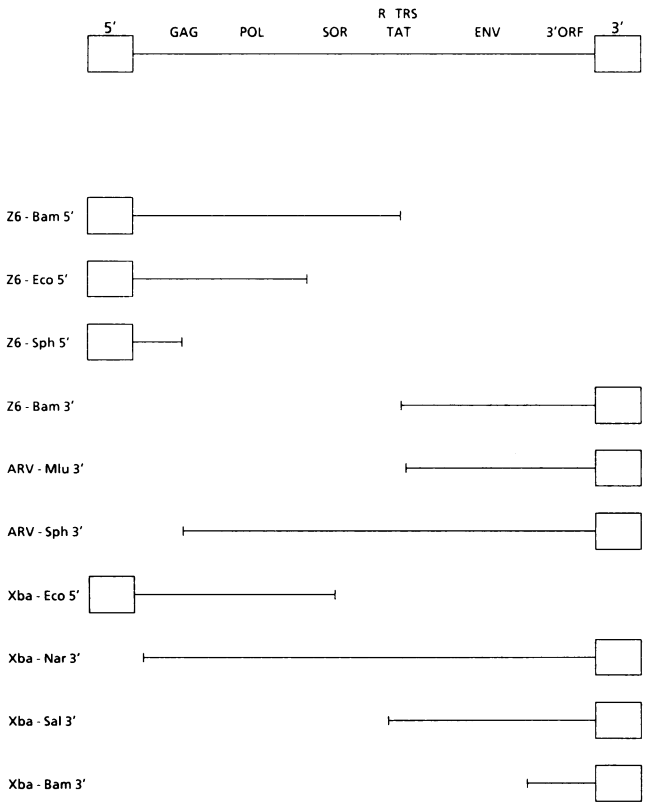

FIG. 3. Structures of viral DNAs used in this study. Parental plasmids containing complete proviral DNA have been described. Detailed restriction maps for pARV and pXba clones were from Weiss *et al.* (47) and pZ6Neo from Srinivasan *et al.* (37). Deletions in the viral DNA were made by taking advantage of the specific restriction enzyme cleavage sites present in the viral DNA and also in the vector DNA sequences. Deletion points in the viral DNA are indicated by the restriction enzyme cleavage site used for construction. All of the deletion constructs were further confirmed by restriction enzyme analysis. Plasmid DNA was prepared as described (44) and banded twice in cesium chloride. DNA (10 μg) was used for transfection into RD cells. Medium was tested for the presence of virus by RT assay (2) and antigen assay (43) at 72 hr after transfection. SOR, *vif*; R, *vpr*; TRS, *rev*; 3'ORF, *nef*.

generating proviral DNA in an expressible form. To resolve this question, HIV released from the cells after transfection of plasmids were checked for infectivity with phytohemagglutinin-stimulated PBLs. PBLs after infection were followed for 12 days by RT assay. Table 3 shows that the viruses derived from pairs with overlapping sequences could productively infect PBLs. These results clearly indicate that recombination had occurred between transfected viral DNAs, generating a complete virion RNA.

Genetic Structure of the Recombinant Viruses. The physical evidence for recombination was obtained by Southern hybridization analysis. PBLs were infected with viruses derived from RD cells cotransfected with truncated HIV clones. High-molecular-weight DNA, extracted from the PBLs 7 days after virus infection, was digested with *Sac* I and hybridized to a full-length viral DNA probe (37). pZ6Neo and pXba proviral DNA show cleavage sites for *Sac* I enzyme only in the LTR sequences or LTR and an additional site close to the LTR, respectively. However, pARV proviral DNA shows an internal *Sac* I site upstream of *env* gene in addition to the sites present in LTR. Taking advantage of this, all of the recombinant viruses were analyzed. As expected, recombinant viruses derived from the pair, Z6-Bam 5' and ARV-Sph 3' which have an overlap homology of 4 kilobase pairs, showed an internal *Sac* I site (Fig. 4). Experiments

Table 1. Recombination between viral DNA fragments

5' viral DNA fragment	3' viral DNA fragment	Virus antigen, OD unit(s)
Z6-Bam 5'	ARV-Sph 3'	1.375
	ARV-Mlu 3'	0.058
	Xba-Sal 3'	0.580
	Xba-Nar 3'	1.685
	CDC 451 3'	0.786
Z6-Eco 5'	ARV-Sph 3'	0.906
	ARV-Mlu 3'	0.037
	Xba-Sal 3'	0.036
	Xba-Nar 3'	1.690
	CDC 451 3'	0.849
Z6-Sph 5'	ARV-Sph 3'	0.044
	ARV-Mlu 3'	0.040
	Xba-Sal 3'	0.040
	Xba-Nar 3'	0.107
	CDC 451 3'	0.043
Xba-Eco 5'	ARV-Sph 3'	0.044
	ARV-Mlu 3'	0.056
	Xba-Sal 3'	0.043
	Xba-Nar 3'	0.044
	CDC 451 3'	0.045

RD cells were cotransfected with 10 μg of each plasmid DNA. Culture supernatant was centrifuged; the virus pellet was dissolved in 200 μl of solubilizing buffer, and a 10-μl aliquot was used for virus antigen assay. Cut-off value in the assay was 0.090 OD units.

done with viruses from three different transfections showed similar results, indicating that restriction enzyme sites in the viral DNA results from recombination between truncated plasmid DNAs. Because hybrid viruses can result from recombination between DNA substrates at multiple sites in the region of homology, further detailed genetic analysis requires plaque purification of hybrid viruses (50). Interestingly, the pair, Z6-Bam 5' and Xba-Sal 3' with an overlap homology of 53 base pairs with multiple point mutations also produced viral particles upon transfection. The genetic structure of hybrid viruses derived from this pair is shown in Fig. 5.

DISCUSSION

The experiments described in this study indicate the ability of cellular machinery to recombine the truncated HIV DNAs to generate complete proviral DNA. The antigen assay we used monitors the release of viruses in the culture medium, which may contain both nonviable virus due to illegitimate recombination and viable virus due to homologous recombination. Our results are similar to the data reported for pairs of noninfectious clones of spleen necrosis virus and nontransforming mutants of polyoma virus (23, 24, 26). The truncated HIV clones used intrinsically lack the ability to produce viral particles that can be detected by antigen assay. HIV antigen assay has been shown more sensitive by several orders of magnitude than RT assay (51). This difference is also evident in our study, as only the antigen assay could detect the low

Table 2. Recombination between exogenous and stable cellular HIV DNA in RD cells

DNA used for transfection	Posttransfection HIV antigen, OD unit(s)	
	Day 3	Day 5
None	0.044	0.057
Z6-Bam 5' (10 μg)	0.244	0.358
Z6-Bam 5' (15 μg)	0.344	1.045

Resident HIV DNA was ARV-Sph 3'.

Table 3. Replication of HIV (derived from transfection of complete or mixture of partial proviral clones) in phytohemagglutinin-stimulated PBL

Virus	Transfection DNA	Postinfection RT activity, cpm per ml of culture fluid		
		6 days	9 days	12 days
Z6	pZ6Neo	201,345	147,905	79,893
ARV	pARV	11,845	78,800	59,480
HIV-R1	Z6-Bam 5′ + ARV-Sph 3′	313,530	378,585	303,745
HIV-R2	Z6-Eco 5′ + ARV-Sph 3′	238,135	143,555	63,433
HIV-R3	Z6-Bam 5′ + Xba-Nar 3′	423,895	321,460	226,795
HIV-R4	Z6-Eco 5′ + Xba-Nar 3′	348,400	391,500	378,505
HIV-R5	Z6-Bam 5′ + Xba-Sal 3′	119,315	103,725	38,588

RT assay was done as described (2).

levels of virus produced by the complete proviral genome generated due to recombination.

Recombination between 5′ and 3′ viral clones released biologically infectious viruses. The viruses recovered from RD cells were successfully transmitted to HUT 78 cells, a cell line susceptible to HIV infection and also to PBLs. Studies reported earlier with selectable marker genes and simian virus 40 indicate the occurrence of both homologous and nonhomologous recombination (21–34). Given the genetic variability of HIV isolates, it is expected that all the products of illegitimate recombination may not be viable because of the disruption of essential protein coding regions. Because the assay used depends upon release of viral particles, homologous recombination may be the predominant mode of joining viral DNA fragments. This conclusion is supported by the following: (*i*) Recombination is dependent on the homology between two transfected plasmids as seen in other systems. (*ii*) Many of the plasmid constructs do not have the proper sequences to generate HIV-specific mRNA. The presence of infectious viral particles in the medium of the transfected cells suggests the presence of complete virion RNA. It is also to be noted that both *tat* and *art/trs* [*rev*, according to new HIV gene nomenclature (52)] gene products are essential for the genesis of mRNA and also for the synthesis of gag and env proteins (53–56)]. The mRNA for *tat* and *art/trs* can be generated only by recombination of 5′ and 3′ truncated clones. (*iii*) The pXba clone is inactive in virus production even though transactivation function was seen. Cotransfection of the deletion mutants derived from Xba clones, Xba-Sal 3′ and Xba-Nar 3′, along with mutants of other proviral clones released viral particles. (*iv*) The viruses, released upon transfection of truncated subclones of HIV,

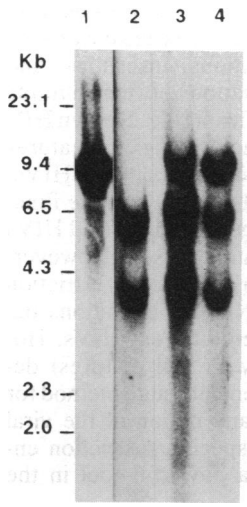

FIG. 4. Southern hybridization analysis of high-molecular-weight DNA extracted from virus-infected cells. Viruses released into the medium, due to the recombination of viral DNA fragments, were pelleted at 35,000 rpm using SW41 rotor and transmitted to phytohemagglutinin-stimulated PBL as described. High-molecular-weight DNA was extracted 7 days after infection. DNA (10 μg) was digested with *Sac* I, electrophoresed on 0.8% agarose gel, and hybridized to nick-translated full-length HIV probe. Lanes: 1, HIV_{Zr6}; 2, HIV_{SF-2}; 3 and 4, hybrid viruses resulting from recombination between Z6-Bam 5′ and ARV-Sph 3′ DNA fragments.

appear to be recombinant in origin because they show distinct bands in Southern hybridization analysis depending on the input DNA.

The mechanism(s) by which cellular enzymes accomplish homologous exchange is not clear. Studies involving marker and viral genes have led to the formulation of the unwinding model (34), double-strand-break repair model (57), and polar stripping model (31) to account for homologous recombination between DNAs in eukaryotic cells. Double-strand breaks are known to stimulate homologous recombination in animal cells (30). Similar double-strand breaks are introduced naturally into transfected DNA *in vivo* (33). DNA breakage and reunion can occur through double-strand cut or single-strand nicking, and action of 5′ → 3′ exonuclease could initiate and allow the crossing over to take place. Alternatively, gene conversion, i.e., nonreciprocal transfers of genetic information may also contribute to the generation of recombinant viruses.

The experiments described here demonstrate that recombination between different HIV molecules *in vivo* is possible. The extent of homology at the DNA sequence level between HIV isolates makes it harder to identify such recombination events. Recently, however, sequence analysis of a number of HIV-1 isolates revealed such a recombination event in an African isolate $HIV\text{-}1_{MAL}$ (58).

HIVs have been shown to exhibit differences in cellular tropism, replication, and cytopathic effects, and the recent data indicate extensive genetic heterogeneity of HIV within

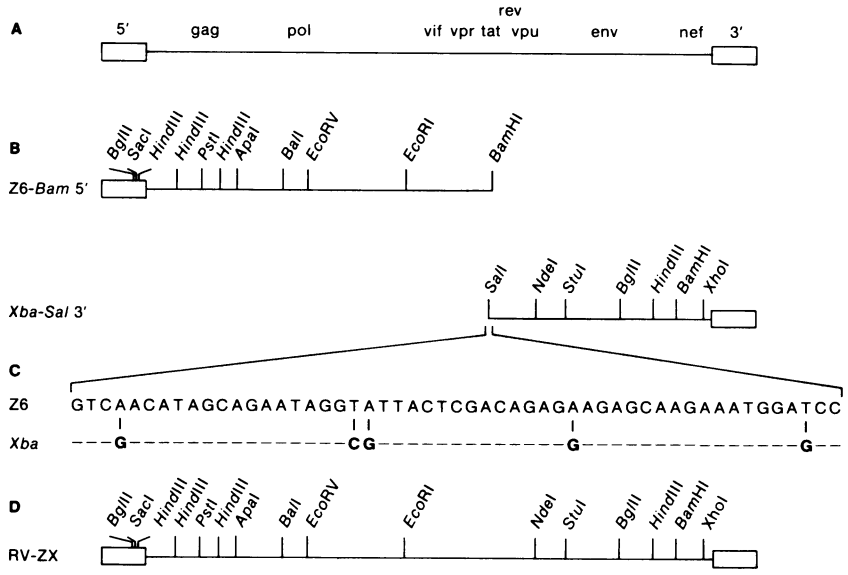

FIG. 5. Restriction enzyme analysis of hybrid virus. (*A*) Diagram of genes in complete proviral DNA. (*B*) Truncated viral DNAs used for transfection to generate hybrid virus. (*C*) Nucleotide sequence at the region of homology between the truncated viral DNAs. Differences in the sequence are indicated in boldface letters. (*D*) Restriction enzyme analysis of hybrid virus designate RV-ZX.

the infected individual (59, 60). The evidence presented in this report indicates that for occurrences of extensive genetic diversity, in addition to point mutations, insertions, and deletions, the events such as recombination between multiple viral sequences may have a very significant role. Known HIV isolates with conditional and distinct biological features should be useful in analyzing recombination between HIVs. Thus, the nature of viral determinants underlying the functional differences can be analyzed by generating hybrid HIVs and comparing them with the parental viruses. However, construction of hybrid HIVs based on compatible restriction enzyme sites present in the viral DNA has limitations because of extensive variability between different HIVs. Homologous recombination methods (with cell cultures) described in this report provide an efficient alternate method for generating hybrid viruses involving any region of the viral genome without the requirement of specific restriction enzyme sites. This technique will be a powerful tool in the analysis of viral gene functions.

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