JOURNAL OF VIROLOGY, Sept. 1991, p. 4847–4852
0022-538X/91/094847-06$02.00/0
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Vol. 65, No. 9

Generation of Hybrid Human Immunodeficiency Virus Utilizing the Cotransfection Method and Analysis of Cellular Tropism

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Received 9 October 1990/Accepted 4 June 1991

Human immunodeficiency viruses (HIV) isolated from infected individuals show tremendous genetic and biologic diversity. To delineate the genetic determinants underlying specific biologic characteristics, such as rate of replication, cytopathic effects, and ability to infect macrophages and T4 lymphoid cells, generation of hybrid HIV using viruses which exhibit distinct biologic features is essential. To develop methods for generating hybrid HIV, we constructed truncated HIV proviral DNA plasmids. Upon digestion with restriction enzymes, these plasmid DNAs were cotransfected into human rhabdomyosarcoma cells to generate hybrid HIV. The hybrid HIVs derived by this method were infectious upon transmission to both phytohemagglutinin-stimulated peripheral blood lymphocytes and established human leukemic T-cell lines. The virus derived from molecular clone pHXB2 ($HIV_{HTLV-III}$) productively infected CEMx174 cells. On the other hand, molecular clone pARV (HIV_{SF2})-derived virus did not show productive infection of CEMx174 cells when used as a cell-free virus. The hybrid HIV containing the 3′ end of the genome from pARV and the 5′ end of the genome from pHXB2 was effective in infecting CEMx174 cells, but the converse hybrid containing 5′ pARV and 3′ pHXB2 was not effective in infecting CEMx174 cells. These results suggest that differences in the genes outside of *env* and *nef* play a role in the ability of the virus to infect a certain cell type. The intracellular ligation method should be useful in the analysis of related and unrelated HIV-1 isolates with common restriction enzyme cleavage sites.

Human immunodeficiency virus type 1 (HIV-1) infects a variety of target tissues, including both hemopoietic cells and the central nervous system (8, 19, 32). Biologically and molecularly distinguishable variants of HIV have been isolated from infected individuals; a comparison of the nucleotide sequences of different HIV-1 isolates revealed variations of 5 to 8% in the long terminal repeat (LTR) sequences and 10 to 15% in the envelope gene (5, 18, 29, 41, 45). Viruses isolated from individuals over a period of time also exhibit sequence divergence and also vary in biological properties (4, 9, 18, 26, 36, 43); virus from asymptomatically infected individuals shows low replication and weaker cytopathic effects. HIV isolates with a high replication rate and syncytium-inducing capacity were more frequently observed in individuals with AIDS or AIDS-related complex (9, 43). The bases for tissue specificity and for the changes from avirulence to virulence of HIV-1 are not known.

The identification of viral genetic determinants that control the distinct biologic features of HIV, such as tropism and virulence, are of fundamental importance to an understanding of the basic molecular mechanisms of HIV-1-associated diseases (15, 20, 30, 31, 34). Viral tropism is readily determined in cells in vitro, depending on the abortive or productive infection based on virus receptors and postpenetration events (28). With this approach, Yourno et al. (49) constructed a chimeric HIV genome in vitro between a North American (HXB2) and a Zairian (JY-1) molecular clone. Studies with viruses derived from the parental and hybrid molecular clones indicated that *vif* and *env* regions play an important role in differential cell tropism. Also, a hybrid molecular clone of HIV-1, constructed from *env* fragments derived from multiple distinguishable clones obtained from a single patient isolate, showed marked differences in infectivity for peripheral blood mononuclear cells, $CD4^+$ lymphocyte lines, and monocyte-macrophages (12). Recent studies by York-Higgins et al. (48) and Cheng-Mayer et al. (2) used intracellular ligation processes to construct hybrid HIVs and showed that the *env* region of the viral genome is responsible for replication in specific cell types and cytopathicity characteristics. Previously, we have shown that hybrid HIV can be generated by homologous recombination between viral DNA fragments transfected into mammalian cells (22, 39, 40). Viruses recovered from the transfected cells were able to grow in phytohemagglutinin-stimulated peripheral blood lymphocytes (PBL), indicating their infectious nature. Since the truncated viral DNA fragments lack the ability to synthesize viral particles independently, the viral particles released into the medium could only result from the proviral DNA generated in vivo by homologous recombination. The limitation of this method, however, is lack of knowledge about the recombination junctions in the viral DNA. To overcome this, we used an alternate method to generate hybrid HIV which is based on the ability of cells to ligate DNA both accurately and efficiently. Since both related and unrelated HIV-1 isolates show a number of conserved restriction enzyme cleavage sites, these sites can be used to construct hybrid HIV intracellularly. The experiments reported here describe the generation of hybrid HIV from $HIV_{HTLV-III}$ and HIV_{SF2} and its ability to infect $CD4^+$ T-lymphoid cell lines.

MATERIALS AND METHODS

Cells. A human rhabdomyosarcoma (RD) cell line (obtained from the American Type Culture Collection) was maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and L-glutamine (540 μg/ml) at 37°C with 5% CO_2. Jurkat, CEMx174, SupT1, and MT-4 cells were maintained as suspension cultures in RPMI 1640 medium; phytohemagglutinin (10 μg/ml)-stimulated PBL were

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grown in RPMI 1640 medium containing T-cell growth factor (10%).

Plasmids. HIV DNA plasmids designated pARV and pZ6Neo were derived from cells infected with HIV_{SF2} and HIV_{Zr6}, respectively (24, 36); plasmids pHXB2 and pXb were both derived from human T-cell lymphotropic virus type III-infected cells (10, 40). 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 (see Fig. 1) (33, 40). The subgenomic plasmid molecular clones were verified by restriction enzyme mapping analysis. In all constructs, either the 5′ or the 3′ LTR was retained.

Transfection. RD cells were split 24 h before transfection, and the growth medium was replaced 1 to 2 h before addition of calcium phosphate-precipitated DNA (17). Cells (10^6) were exposed to the precipitate for 8 h, followed by a 90-s glycerol shock (14).

Virus infection. For virus infection studies, culture supernatant from transfected or infected cells was initially filtered through a 0.22-μm-pore-size membrane. Cells (2×10^7) were mixed with the equivalent of 100 ng of p24 from cell-free virus for 1 to 4 h. The cells were then thoroughly washed to remove the virus inoculum and suspended in RPMI 1640 medium at a concentration of 10^6/ml. Aliquots were taken at 3-day intervals, and the viral particles released into the medium were quantitated by viral p24 antigen assay.

HIV antigen assay. The HIV antigen assay (Coulter) was performed as recommended by the manufacturer to quantitate the amount of virus (16).

Southern hybridization. Parental and recombinant viruses were propagated in phytohemagglutinin-stimulated PBL as previously described (36). High-molecular-weight DNA was extracted from cells 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 a nick-translated full-length HIV probe (36).

Polymerase chain reaction (PCR). High-molecular-weight DNA was extracted from cells by the method described by Srinivasan et al. (38). PCR was performed by utilizing a *gag* gene-specific primer pair. The primers 5′-ATAATCCACC TATCCCAGTAGGAGAAAT-3′ (plus strand) and 5′-TTTG GTCCTTGTCTTATGTCCAGAATGC-3′ (minus strand) resulted in generation of a 115-bp fragment. Typically, the reaction was carried out with 1 μg of DNA in a total volume of 100 μl involving 35 cycles of amplification. The reaction products were run on a 1.5% agarose gel. The DNA fragments were transferred to nitrocellulose filters and hybridized to the end-labeled oligonucleotide probe 5′-ATCCT GGGATTAAATAAAATAGTAAGAATG-3′ as previously described (6). To verify the identity of U3 sequences in the LTR regions of the hybrid viruses, DNAs obtained from parental and hybrid virus-infected cells were subjected to PCR with the primers 5′-CCAGTCACACCTCAGGTAC CT-3′ (plus strand; located 84 bp upstream of the 3′ LTR) and 5′-GGTCTGAGGGATCTCTAG-3′ (minus strand; 32 bp from the end of the 3′ LTR). These primers resulted in generation of a 690-bp fragment from the 3′ end of the proviral DNA, which was purified on agarose gel for further analysis. A similar approach was used to amplify the 5′ end of the proviral DNA with appropriate primers (5′-GCTA ATTTGGTCCCAAAGA-3′ [plus strand] and 5′-TCTGCA GCTTCCTCATTGAT-3′ [minus strand]), which resulted in generation of a 1.45-kbp DNA fragment.

Restriction enzyme mapping. PCR-generated DNA fragments were purified on agarose gel and labeled with T4 polynucleotide kinase. Mapping was done with the partial- and double-digestion methods (33).

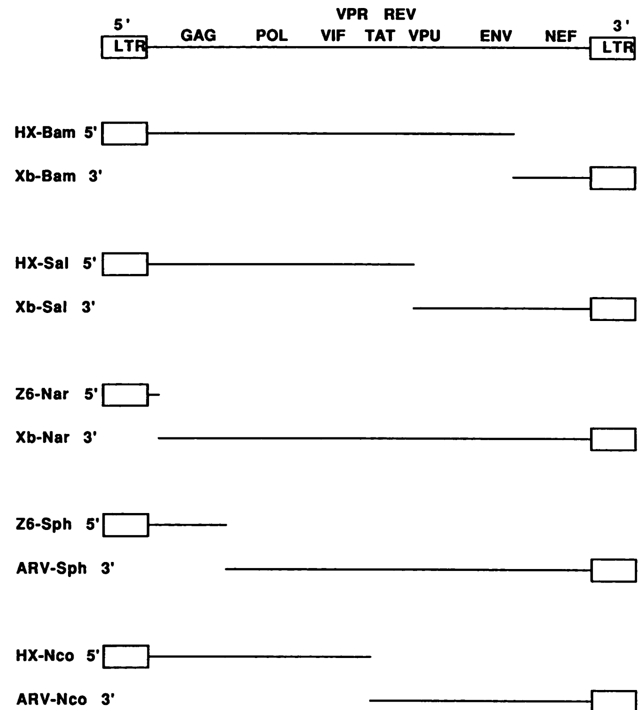

FIG. 1. Structures of the viral DNAs used in this study. Parental plasmids containing complete proviral DNAs (pHXB2, pXb, pARV, and pZ6Neo) have already been described (38). Deletions in the proviral DNA were made by taking advantage of the specific restriction enzyme cleavage sites present in the viral DNA. Deletion points in the viral DNA are indicated by the restriction enzyme cleavage site used for construction. The subgenomic clones of each proviral DNA are indicated, with the designation of the proviral DNA clone followed by the restriction enzyme used for cloning. HX, pHXB2; Xb, pXb; Z6, pZ6Neo; ARV, pARV.

RESULTS

Construction of truncated HIV proviral DNA and generation of hybrid HIV by cotransfection. The proviral DNAs designated pZ6Neo, pARV, and pHXB2 have been shown to produce measurable amounts of virus upon transfection into human RD cells (37, 40). The clone designated pXb was molecularly cloned from the DNA obtained from human T-cell lymphotropic virus type III-infected H9 cells and found to produce a low level of virus. This proviral DNA has been shown to harbor a defect in the 5′ end of the genome. However, the 3′ end of proviral DNA serves as an efficient substrate to generate hybrid HIV in combination with other truncated proviral DNAs (35, 40). Convenient restriction endonuclease cleavage sites, such as *Xho*I, *Sph*I, *Nco*I, *Sal*I, and *Nar*I, present in the viral and vector DNAs were used to produce the truncated proviral DNA plasmids. The structures of the truncated proviral DNAs generated are shown in Fig. 1. The modified proviral DNAs have either 5′ or 3′ LTRs and a portion of the viral genome. The modified proviral DNAs were tested for biologic activity upon transfection into RD cells. Culture supernatants were analyzed sequentially for the presence of viral particles by viral antigen assay. As expected, all of the modified proviral

TABLE 1. Generation of hybrid HIVs by transfection of truncated proviral DNAs into RD cells[a]

5′ proviral DNA	3′ proviral DNA	Concn (pg/ml) of p24 antigen released into medium after transfection[b]	
		72 h	120 h
Z6-Sph	ARV-Sph	11,359.0	5,578.0
HX-Sph	ARV-Sph	923.0	497.0
Z6-Nar	Xb-Nar	56.0	55.8
HX-Sph	Xb-Sph	167.0	244.0
HX-Sal	Xb-Sal	906.0	178.6
HX-Nco	ARV-Nco	132.0	65.7

[a] The truncated subgenomic clones are described in Fig. 1 (Z6, pZ6Neo; HX, pHXB2; ARV, pARV; Xb, pXb).
[b] RD cells (10^6) were cotransfected with 10 μg of each plasmid DNA digested with the appropriate restriction enzyme by the calcium phosphate precipitation method. Culture supernatant was assayed for p24 antigen level after 72 and 120 h. The cutoff value in the assay was 2.4 pg/ml.

TABLE 2. Infectivities of cell-free parental and hybrid HIVs in CEMx174 cells[a]

Virus used for infection	Concn of p24 antigen (pg/ml) at postinfection day:				
	6	8	10	12	15
pARV	—	—	—	—	—
pHXB2	507.5	15,215	213,000	536,000	840,500
pHA1	—	—	—	—	—
pHA2	103.18	640.05	ND	16,916.6	385,333
pAH1	—	—	—	—	—

[a] The details of the hybrid HIVs used are described in Fig. 2. Hybrid HIVs were initially grown in PBL. We used 2×10^7 CEM cells and the equivalent of 100 ng of p24 from cell-free virus for infectivity studies. Culture supernatants of the infected cells were evaluated for the presence of viral particles by HIV p24 antigen assay. —, value below the cutoff level, which was 2.4 pg/ml. All values for days 0, 2, and 4 postinfection were below the cutoff. ND, not determined.

DNAs were inactive in supporting the synthesis of viral particles and offer ideal substrates for construction of hybrid HIV.

It has been shown previously by a number of investigators that eukaryotic cellular machinery is very efficient in ligating the DNA fragments with cohesive and blunt ends (23, 25, 27). Transfection experiments involving DNAs from different molecular clones showed high levels of p24 antigen in the medium (Table 1). The amount of virus production depended on the restriction enzyme site used and the source of the truncated proviral DNA fragment.

Infectivity of hybrid HIV in human PBL and leukemic cell lines. The viral particles released from the cells after cotransfection of plasmid DNA were checked for infectivity with phytohemagglutinin-stimulated PBL. After infection, PBL were monitored by antigen assay for virus production. The virus derived from cotransfection of Z6-Sph 5′ and ARV-Sph 3′ DNA fragments and other combinations of DNAs productively infected PBL, and the antigen values reached 3,000 to 5,000 pg/ml in the PBL (2×10^7) at day 8 after infection. This indicates that intracellular ligation of DNA fragments resulted in generation of complete proviral DNA which directed the synthesis of virion RNA and proteins.

Viruses recovered from cells after transfection with different proviral DNAs show different tropisms for established cell lines such as CEMx174, SupT1, MT-4, and PBL when tested as a cell-free virus. Although all of the viruses were able to replicate in PBL, only viruses derived from clone pHXB2 produced an active infection in established cell lines. The pARV molecular-clone-derived virus showed no evidence of infection in the 15-day period analyzed (Table 2). In accordance with this observation, hybrid virus containing sequences from the 3′ end of the pARV proviral DNA (downstream of the *Sph*I cleavage site, 1,460 bp from the 5′ LTR) did not infect CEMx174 cells. On the other hand, hybrid virus containing the 5′ end of the genome from HXB2 (5,709 bp) and the 3′ end of the genome from pARV was able to replicate in CEMx174 cells (Table 2 and Fig. 2).

Genetic structure of hybrid HIV. PBL at 6 or 7 days after infection were lysed, and high-molecular-weight DNA was extracted and used to carry out PCR utilizing *gag* gene-specific primer pairs of HIV-1. The presence of viral DNA was evident by the appearance of a 115-bp fragment (Fig. 3). The genetic structure of hybrid HIV was verified by restriction enzyme mapping analysis of the U3 sequences of the LTR region. Since it is known that U3 sequences of the LTR region are contributed by the 3′ LTR of the viral DNA, the hybrid HIV should encompass U3 sequences from the 3′ end of the viral DNA fragment used for construction of the hybrid HIV. DNA obtained from the infected cells was used for PCR with appropriate primers for 5′ and 3′ LTRs, and the resulting fragments were tested for cleavage by using *Eco*RV, *Bsa*AI, *Dra*III, *Nci*I, *Tha*I, and *Aha*II restriction enzymes. As expected, hybrid HIV resulting from 5′ pHXB2 and 3′ pARV fragments showed U3 sequences characteristic of pARV proviral DNA (Fig. 4).

DISCUSSION

Somatic cells have been widely used to study the regulation of gene expression involving transfection methods. Recently, somatic cells have also been used to analyze the efficiency of ligation and recombination between DNAs (13, 21, 23, 25, 44, 47). DNAs introduced into the cells by the transfection method have been shown to ligate efficiently. Intramolecular and intermolecular blunt and cohesive ends

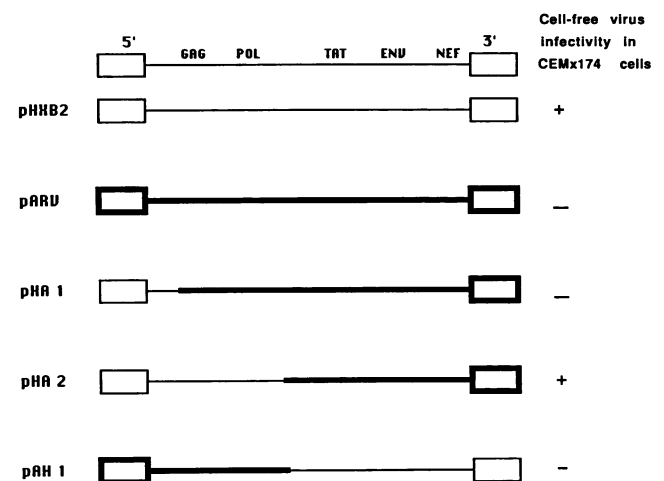

FIG. 2. Structure of hybrid HIVs and their abilities to infect CEMx174 cells. Molecular clones designated pHXB2 and pARV were used to construct truncated HIV proviral DNAs for generation of hybrid HIV. The restriction enzyme cleavage sites used for generation of hybrid HIV were *Sph*I for pHA1, *Nco*I for pHA2, and *Nco*I for pAH1.

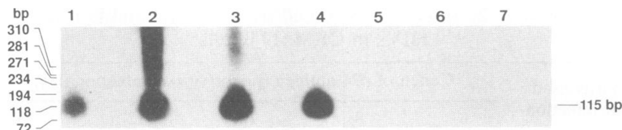

FIG. 3. Analysis of viral sequences by PCR in the high-molecular-weight DNA extracted from PBL infected with hybrid HIV. The *gag* gene-specific primers generate a 115-bp fragment upon amplification. The PCR-amplified fragment was electrophoresed and hybridized by using an end-labeled oligonucleotide probe specific for *gag* (5). Lanes: 1, positive control; 2, pARV-infected PBL DNA; 3, DNA from PBL infected with hybrid viruses resulting from recombination between Z6-Sph 5′ and ARV-Sph 3′ DNA fragments; 4, pZ6-infected PBL DNA; 5, negative control—normal PBL DNA; 6, DNA from cells exposed to the supernatant of cells transfected only with the Z6-Sph 5′ fragment; 7, DNA from cells exposed to the supernatant of cells transfected only with the ARV-Sph 3′ fragment.

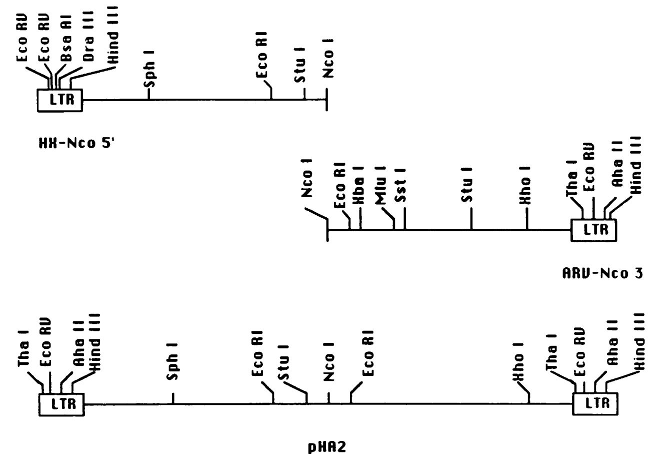

FIG. 4. Genetic structure of hybrid HIV. Restriction enzyme maps of the truncated (HX-Nco 5′ and ARV-Nco 3′) and hybrid proviral (pHA2) DNAs are presented. High-molecular-weight DNA derived from hybrid HIV-infected cells was cleaved with restriction enzymes, transferred to a nitrocellulose filter, and hybridized to a 5′ (*gag*)- or 3′ (*env*)-specific probe as previously described (34). To generate a restriction enzyme map of the U3 region of the LTR, appropriate primers were used for PCR and the amplified DNA was cleaved with different restriction enzymes.

have been shown to ligate equally efficiently. Mertz and Davis (25) demonstrated that the cohesive nature of the termini generated by cleavage of the simian virus 40 genome with restriction enzyme *Eco*RI ligated intracellularly. Plaques were generated at a frequency of 10% of those produced with supercoiled simian virus 40 DNA. The transfection experiments with the different truncated HIV-1 DNAs described here show that the virus production due to intracellular ligation is in the range of 1 to 2% in comparison with the wild-type proviral DNA (data not shown). Miller and Temin (27) noted that coprecipitation of DNA fragments led to production of high levels of infectious virus. Efficient ligation of DNA fragments has been observed in cells transfected with DNA or microinjected DNA. Cell-mediated ligation was exploited to construct hybrid viruses by using subgenomic clones of HIV. Transfection of a single clone or circular plasmid DNA did not lead to release of detectable virus into the culture medium. When cotransfection was carried out with two separate DNA fragments (5′ and 3′) linearized with the same restriction enzyme, release of infectious virus was observed. These results imply that ligation of DNA fragments is a prerequisite step for synthesis and assembly of viral particles. Release of infectious virus also serves as a stringent parameter to eliminate DNA that is damaged during the transfection procedure (46). Since the restriction enzyme cleavage sites are in the regions of viral DNA coding for specific viral proteins (1), modification of or damage to the DNA would abolish the ability to produce infectious virus. Cotransfection of heterotypic viral DNA fragments into RD cells resulted in release of viral particles. The efficiency of virus production, however, seemed to be different (Table 1). This may be due to the differences in the abilities of cells to ligate different restriction enzyme-cleaved DNAs. In addition, it has also been demonstrated that different HIV proviral DNAs showed differential amounts of virus production upon transfection into human RD cells (40). Such differences could very well reflect on the overall virus production when heterotypic viral DNAs are used as substrates for generation of hybrid HIV.

Hybrid HIVs generated by using different molecular clones have been used to analyze the tropism of the virus. In view of the isolation of HIV from different tissues of infected individuals, the tropism of HIV may be an important biologic feature which plays a significant role in the induction of disease in virus-positive individuals. Since HIV tropism cannot be studied in vivo, it is studied in vitro by using normal PBL, glial cells, and established human leukemic cell lines, such as SupT1, Jurkat, CEMx174, and MT-4. Cell-free virus prepared from a transfected and/or infected culture was used as the source of inoculum for virus infection studies. Our experiments with hybrid HIV generated between pHXB2 and pARV molecular clones revealed that failure to infect certain cell types correlates with the region of the genome outside of *nef* and *env* genes. These results are interesting and differ from the data recently reported involving other HIV isolates (2, 48). The *env* gene of the HIV-1 genome was shown to be responsible for the replication and cytopathic effects of HIVs. Similar data were also reported for the macrophage tropism of HIV strains (30, 34). The results obtained in our study, however, can be explained on the grounds that different genes of HIV, either alone or in combination, contribute to the tropism of the virus. It should also be pointed out that previous studies on pARV molecular clone-derived virus used cocultivation methods to evaluate the infectivity characteristics of the virus. Overlaying of cells onto transfected monolayer cells efficiently transmitted the virus to these cells (2, 48). The results of our studies indicate that pARV-derived virus behaves differently as a cell-free virus. The lack of productive infection of CEM cells by the virus derived from the molecular clone designated HIV-1_{SF2MC} (pARV) was also recently reported by Cheng-Mayer et al. (3). Studies by Etkin and Bukrinsky (7) showed functional heterogeneity of the ARV-2 (HIV_{SF-2}) strain that was originally used for the molecular cloning of pARV. Biological cloning of the virus by the limiting-dilution method revealed the presence of several substrains that lack the ability to infect MOLT-4 or MT-4 cells. Such a scenario is also consistent with the multiple mechanisms underlying the tropism of a virus, including attachment of the virus to the receptor and penetration and postpenetration events (28). It is important to point out that the *vif* gene of HIV has been shown to play a key role in the ability of the virus to infect cells as a cell-free virus (11, 42). Our results obtained with hybrids between $HIV_{HTLVIII}$ and HIV_{SF2} suggest that the ability to infect CEMx174 cells as a cell-free virus does

not segregate with the 3′ end of the genome including the U3 region of the LTR. We are in the process of generating a number of hybrid viruses involving pHXB2 ($HIV_{HTLV-III}$) and pARV (HIV_{SF2}) proviral DNAs to address the mechanisms underlying lack of infection of CEMx174 cells by virus derived from the pARV molecular clone.

Intracellular ligation of DNA fragments is an efficient method with which to construct hybrid DNA molecules. The parameter we used in our experiment is release of infectious HIV particles directed by HIV DNA upon ligation. Studies with other retroviruses showed that the recombinant-virus approach led to identification of disease specificity and tropism determinants within the LTR and *env* gene sequences. The advantages of generation of a hybrid virus through the restriction site-dependent method are severalfold. (i) This method depends on the intracellular ligation which occurs most efficiently and accurately. (ii) This method relies on the presence of conserved restriction enzyme cleavage sites which are present in both related and unrelated viral isolates. (iii) This method eliminates the need for reconstruction of hybrid proviral DNA in vitro involving time-consuming genetic manipulation techniques. (iv) Unlike the homologous recombination method, hybrid viruses generated through the restriction site-dependent method have precisely known recombination junctions. The method described in this report to generate hybrid HIV will be useful in the analysis of genetic determinants underlying distinct biologic features of HIV.

ACKNOWLEDGMENTS

We thank the Wistar Institute editorial staff for preparation of the manuscript. We gratefully acknowledge R. C. Gallo, F. Wong-Staal, and P. A. Luciw for providing proviral DNAs derived from $HIV_{HTLV-III}$ and HIV_{SF2}, respectively, and J. Hoxie for CEMx174 cells.

This work was supported by grants AI29306 and AI25380 from the National Institutes of Health.

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