

V3-Independent Determinants of Macrophage Tropism in a Primary Human Immunodeficiency Virus Type 1 Isolate

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Human immunodeficiency virus type 1 isolates differ in their ability to productively infect macrophages, and several groups have mapped the genetic basis for macrophage tropism to regions of *env* that include the third hypervariable region (V3 loop). We recently described a primary isolate (89.6) which is highly macrophage tropic and yet differs from other macrophage-tropic strains studied in that it is cytopathic in T cells. Genetic mapping of macrophage tropism determinants in this virus was done by using chimeras generated with the prototypic non-macrophage-tropic strain HXB2. Replacement of a 2.7-kb *env*-containing region of HXB with corresponding sequences from 89.6 conferred the macrophage-tropic phenotype, but insertion of the 89.6 V3 loop along with V4/V5 sequences did not. Conversely, placement of HXB sequences that included V3 into 89.6 did not impair this strain's ability to replicate in macrophages. Sequence analysis of V3 shows that 89.6 differs markedly from previously described macrophage-tropic consensus sequences and that it is more similar to highly charged non-macrophage-tropic strains. This suggests either that macrophage tropism is defined by structural determinants resulting from complex interactions among multiple *env* regions rather than V3 sequence-specific requirements or that there are multiple mechanisms by which different strains may establish productive macrophage infection. In addition, because the HXB V3 loop supports productive macrophage infection in the background of 89.6, phenotypic characterization of V3 sequences should be considered specific to the viral context in which they are placed.

Human immunodeficiency virus type 1 (HIV-1) isolates vary greatly in their ability to infect and replicate in primary macrophages, and macrophage-tropic strains and infected macrophages are believed to play important roles in HIV pathogenesis. In addition to biological diversity, HIV-1 strains display a remarkable degree of genetic heterogeneity, and several groups have addressed the genetic determinants of macrophage tropism. By using chimeric viruses generated from full or partial clones of macrophage-tropic strains and cloned non-macrophage-tropic isolates, a segment of the *env* gene that contains the third hypervariable region (V3) has been found to be the principal determinant of tropism for macrophages (23, 31, 35, 37, 39). In some studies the V3 region alone appeared to be sufficient to confer macrophage tropism to a non-macrophage-tropic clone (23), while other researchers reported that additional segments were required for maximum efficiency (31, 35, 37, 39).

A striking feature of the V3 loop sequences of those macrophage-tropic viruses is that they are very similar to one another, despite the fact that this domain is one of the most divergent regions among HIV-1 sequences. For that reason, this sequence pattern is considered a signature motif for macrophage-tropic viruses, and detection of this sequence in tissues or in incompletely characterized or uncloned viruses is often used as a surrogate for biological characterization (32, 40). In addition to their genetic similarity, the macrophage-tropic isolates analyzed in this way are biologically similar in

that they do not produce syncytia in lymphocytes (i.e., they are non-syncytium inducing [NSI]). Because the V3 region also contributes to syncytium induction (13, 14, 36), genetic mapping of macrophage tropism performed with these strains may yield results that are specific for this group of isolates. We recently described the isolation and generation of an infectious molecular clone of a primary isolate derived from peripheral blood mononuclear cells (HIV-1/89.6) that replicates efficiently in primary macrophages but differs from prototypic macrophage-tropic strains in that it is extremely syncytium inducing (SI) in T cells (10). Sequence analysis of the V3 region of this isolate revealed that it is highly divergent from those of other well-characterized macrophage-tropic viruses. This indicated that there were multiple V3 patterns compatible with macrophage tropism, and it raised the possibility that alternative regions may be involved in determining tropism.

Because of this, we sought to address the genetic determinants of macrophage tropism in strain 89.6 and to explore the functional consequences of non-macrophage-tropic V3 sequences in the context of this virus. In the present study we constructed chimeric viruses from the biologically active molecular clone of strain 89.6 and the prototypic non-macrophage-tropic cloned strain HXB2. To evaluate the context-dependent effects of specific regions, we utilized both the HXB and 89.6 infectious clones as backgrounds for exchanges. We found that the V3 loop of 89.6 is not a significant determinant of macrophage tropism in this virus. Furthermore, within the background of 89.6, the V3 region of the non-macrophage-tropic virus HXB is fully competent for productive macrophage infection. These results demonstrate that there are alternative V3-independent sequences which enable some naturally occurring viruses to productively infect macro-

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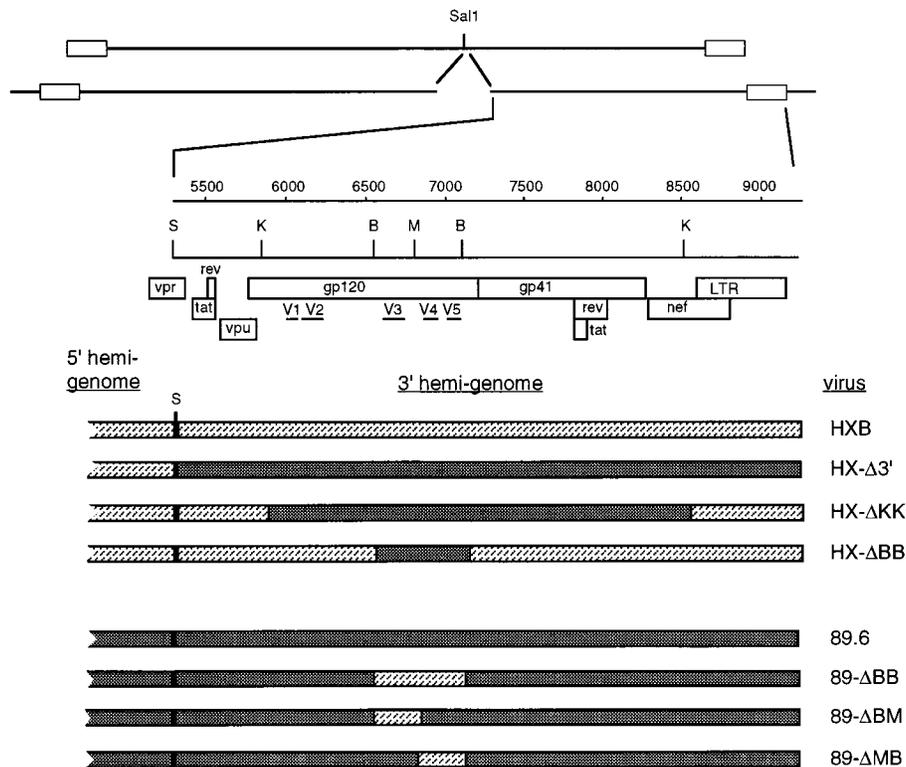


FIG. 1. Viruses used in this study. Chimeric molecules were generated in the 3' hemigenome subclone and then transfected with the corresponding 5' subclone to generate the viruses indicated. The hatched pattern represents HXB-derived sequences, and segments derived from 89.6 are shaded. Restriction sites used to construct the chimeras and their positions relative to important genetic elements are noted. K, *Kpn*I; B, *Bgl*II; M, *Mst*II; S, *Sal*I.

phages and that under some circumstances the V3 region of a prototypic non-macrophage-tropic strain is competent to support macrophage infection. This suggests that complex structural interactions rather than sequence-specific motifs are likely to be critical in the determination of macrophage tropism.

MATERIALS AND METHODS

Generation of chimeric viruses. The infectious molecular clones p89.6 and HXB2 were derived from the primary HIV-1 isolate 89.6 and the prototypic strain IIB, respectively (10, 34). To generate recombinants, the 5' and 3' halves of each proviral clone were subcloned independently by utilizing a unique *Sal*I site at nucleotide 5332 (according to the numbering of HXB2 [30]) that was shared by both clones. Chimeric molecules were generated within the 3' subclone by using shared restriction sites (Fig. 1) and standard subcloning techniques. Each recombinant was verified by restriction analysis followed by direct sequencing across at least one site and generally both sites of recombination. To generate chimeric viruses, equal amounts of 5' and 3' subclone DNA were digested with *Sal*I to produce compatible termini and cotransfected into CD4-negative RD cells by the calcium phosphate method as previously described (2). Virus was then amplified in CEMX174 cells, and virus stocks were harvested at the time of peak viral antigen production (generally 7 to 14 days after transfection), filtered (0.45- μ m pore size), and stored at -80° until use. Virus stocks were quantified by p24 antigen level or 50% tissue culture infective dose content as determined by endpoint dilution on CEMX174 cells.

Cell maintenance and infections. Monocyte-derived macrophages (MDM) were isolated from peripheral blood mononuclear cells of seronegative donors by a two-step adherence procedure as previously described (11). Cells were plated at 2×10^5 to 4×10^5 cells per well in 24- or 48-well plastic tissue culture plates and maintained for ~ 7 days prior to infection to allow differentiation into macrophages, and the medium was changed every 3 to 5 days. Cultures were maintained in Dulbecco modified Eagle medium containing 20% fetal bovine serum, glutamine (2 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml), and they were supplemented with macrophage colony-stimulating factor (100 U/ml; Genetics Institute, Cambridge, Mass.). Peripheral blood lymphocytes (PBL) were derived from periph-

eral blood mononuclear cells by serial depletion of adherent cells and were maintained in RPMI medium supplemented with 20% fetal bovine serum, glutamine (1 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). PBL were stimulated with phytohemagglutinin (5 μ g/ml) for 3 to 4 days prior to infection and maintained thereafter with interleukin-2 (20 U/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). CEMX174 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum, glutamine (1 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). Cells were infected with equivalent amounts of virus equalized on the basis of either p24 antigen content or 50% tissue culture infective dose value. Inoculations were carried out for 4 to 16 h at 37° C, after which cells were washed three times with phosphate-buffered saline (PBS) and refed. The medium was replaced (MDM) or the cells split (PBL) every 3 to 5 days, and the supernatant was harvested for p24 antigen determination by enzyme-linked immunosorbent assay (Coulter Inc., Hialeah, Fla.).

Restriction analysis of recombinant viruses. To verify that the viruses employed in the infections corresponded to the chimeric clones, CEMX174 cells were infected with virus stocks and when cytopathic effects were seen, approximately 5×10^5 to 10×10^5 cells were washed with PBS and suspended in 100 μ l of DNA lysis buffer (100 mM KCl, 20 mM Tris [pH 8.4], 0.1% Nonidet P-40, 500 μ g of proteinase K per ml), and the suspension was incubated for 2 h at 60° C and then boiled for 15 min. PCR amplification was then performed on 5 μ l of lysate by using primers 8227 (nucleotides 8227 to 8246; 5'-TAGCTGAGGGGACA GATAGG-3') and 9186 (nucleotides 9186 to 9164; 5'-CACACTACTTGA AGCACTCA-3') and primers 6541 (nucleotides 6541 to 6563; 5'-ACTCAACTGCTGTAAATGGC AG-3') and 7144 (nucleotides 7144 to 7121; 5'-ATCTCTTGTAGTAGCAGC CCTG-3'), which span the V3 and V4/V5 regions. Amplification was carried out by using *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.) under standard conditions. After amplification, 5 to 10 μ l of product was digested with restriction enzymes that cut within the amplified region of recombination in only one of the two parental clones and analyzed by agarose gel electrophoresis. Sites utilized were *Dra*I (nucleotide 6795; unique to HXB within the V3 *Bgl*II-*Mst*II region), *Afl*III (nucleotide 7044; unique to HXB within the V4/V5 *Mst*II-*Bgl*II region), *Bpu*11021 (nucleotide 8410; unique to HXB within the 2.7-kb *Kpn*I-*Kpn*I *env* exchange), and *Acc*I (nucleotide 8683; outside the exchanged regions and unique to 89.6).

RESULTS

Parental viruses and construction of recombinant proviral clones. HIV-1/89.6 is a macrophage-tropic primary isolate that was obtained from peripheral blood mononuclear cells of an individual with AIDS, and the full-length infectious molecular clone yields virus that is indistinguishable from the uncloned primary isolate in replication kinetics, host cell tropism, cytopathogenicity, and other characteristics (10). For genetic mapping of tropism determinants we selected the infectious molecular clone HXB2 of the non-macrophage-tropic strain IIIB (34), since this is a well-characterized prototype that has been utilized extensively for in vitro studies. Both strains replicate rapidly and to high titers in primary PBL and in the T-cell-B-cell hybrid CEMX174 cell line, and both result in syncytium formation and cytopathic effects. When inoculated onto primary macrophages, strain 89.6 replicates to high titers, while HXB replicates poorly or not at all.

To determine whether tropism was linked to the 3' end of 89.6, the entire 3' region of 89.6 was placed into the HXB background by cotransfection of the 5' subclone of HXB and the 3' subclone of 89.6 to produce HX- Δ 3' (Fig. 1). For analysis of the whole *env* region, we constructed HX- Δ KK by using shared *Kpn*I sites (nucleotides 5897 and 8565). This construct has an HXB background with ~2.7 kb of sequences derived from 89.6 that included all of *env* except for the signal peptide region and first 12 residues of the mature protein, as well as the second exons of *tat* and *rev* and the 5' portion of *nef*. To address the role of specific *env* regions, a pair of reciprocal recombinants was constructed, in which a 580-bp fragment flanked by *Bgl*II sites (nucleotides 6587 and 7167) coding for 193 amino acids of the V3, V4, and V5 domains of gp120 were transferred between viruses to produce HX- Δ BB and 89- Δ BB. An additional pair of recombinants in which regions containing the V3 and V4/V5 domains of HXB were inserted independently into the 89.6 background was then generated; 89- Δ BM contains a 275-bp region of HX-derived sequences flanked by *Bgl*II and *Mst*II sites (nucleotides 6587 and 6862, respectively) that encode 91 residues including V3, and 89- Δ MB includes a 305-bp *Mst*II-*Bgl*III fragment (6862 and 7167) from HXB that codes for 101 residues of V4 and V5. Chimeric molecules were constructed in the 3' subclones, which were then cotransfected with the corresponding 5' subclones to generate infectious virus (Fig. 1). For consistency, the wild-type HXB and 89.6 parental viruses used in these experiments were generated in parallel by cotransfection of wild-type 5' and 3' subclones. Infections were carried out by using inocula equilibrated on the basis of either p24 antigen or 50% tissue culture infective dose content, and results were similar with both methods (data not shown). Several other recombinants were generated, resulting in viruses that were replication defective or attenuated in T cells, but to address macrophage-specific determinants only those which displayed fully permissive replication patterns in both PBL and CEMX174 cells were utilized for this analysis of macrophage tropism.

Replication of chimeric viruses in primary macrophages. All of the viruses reported here displayed wild-type replication kinetics in PBL and CEMX174 cells (data not shown). Replacement of the entire 3' region of HXB with sequences derived from 89.6 resulted in a virus (HX- Δ 3') that possessed all of *env* derived from 89.6, as well as *vpu*, *nef*, the second exons of *tat* and *rev*, and the 3' region of *vpr*. Because of the nature of reverse transcription, U3 would be derived from 89.6, while R and U5 would carry HXB-derived sequences. As expected, HX- Δ 3' replicated efficiently in MDM (Fig. 2A and Table 1), indicating that macrophage tropism in 89.6 was

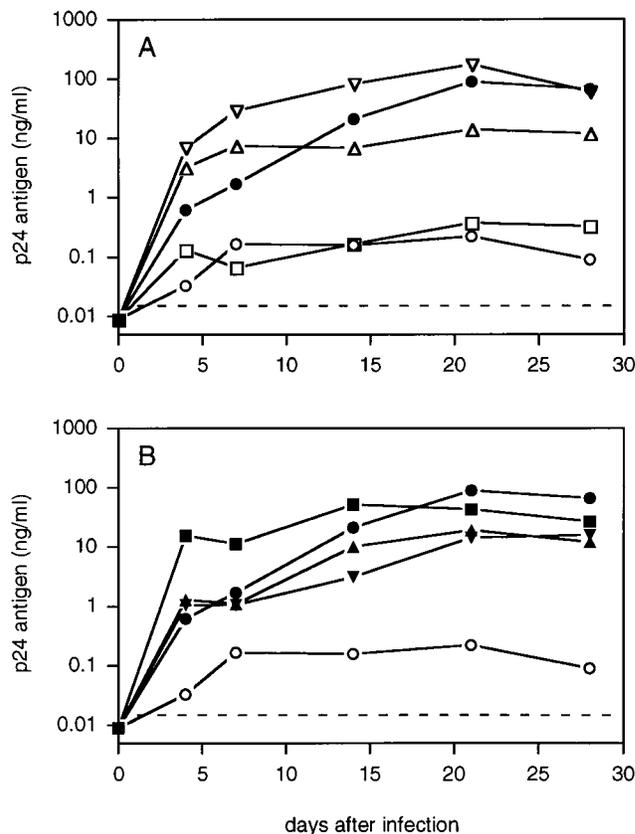


FIG. 2. Replication of chimeric viruses in primary MDM. Seven-day-old cultures of MDM were inoculated with equal amounts of virus (200 ng of p24 antigen) as described in Materials and Methods and washed extensively, and the supernatant was sampled periodically to determine p24 antigen production. (A) HXB-based viruses in which regions were replaced with corresponding sequences from 89.6. (B) 89.6-based viruses in which regions were replaced with corresponding sequences from HXB. \circ , HXB; \bullet , 89.6; ∇ , HX- Δ 3'; Δ , HX- Δ KK; \square , HX- Δ BB; \blacksquare , 89- Δ BB; \blacktriangledown , 89- Δ BM; \blacktriangle , 89- Δ MB.

linked to the 3' end of the genome. While HX- Δ 3' has 89.6-derived *vpu*, *tat*, *rev*, and *nef* sequences in addition to *env*, it is predicted to encode a truncated *vpr* (Table 2), since HXB2 has a frameshift mutation in *vpr* located upstream of the *Sal*I site used for recombination. Therefore, the finding that replication of HX- Δ 3' in MDM was quantitatively similar to that of wild-type 89.6 was somewhat unexpected, since we and others have previously reported that *vpr* is necessary for maximal replication in primary macrophages (2, 3, 38).

To address the effect of *env* exchange, MDM were infected with HX- Δ KK, which carries all of the 89.6 envelope except for 12 N-terminal residues. HX- Δ KK established productive infection of MDM (Fig. 2A and Table 1), indicating that the sequences of 89.6 responsible for macrophage tropism were contained in this 2.7-kb fragment. In addition to *env*, this exchange inserted 89.6 sequences that included the second exons of *tat* and *rev* and the 5' portion of *nef*. Of note, the efficiency of replication was somewhat reduced compared with the parental 89.6 and with the complete 3' exchange, HX- Δ 3'. In most cell donors HX- Δ KK replicated to levels of 10 to 20 ng/ml, which were approximately 5- to 10-fold lower than the levels for 89.6, but in some donors it replicated to only modest levels (Table 1). In all cases, however, levels for this strain were higher than those for the parental HXB. Interestingly, HX- Δ KK has *vpr*, *vpu*, and 3' *nef* genes derived from HXB, which results in

TABLE 1. Replication of chimeric viruses in macrophages^a

Virus	Peak p24 ^{agg} antigen level (ng/ml) ^b									
	Expt A		Expt B		Expt C		Expt D		Expt E	
	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2	Plate 1	Plate 2
HXB	0.45	0.38	0.55	0.57	0.221	0.135	0.127	0.119	0.052	0.267
HX-Δ3'	430	192	182	157	175	70	66	29.8	25.0	180
HX-ΔKK	ND ^c	ND	ND	ND	14.0	6.6	1.39	1.75	15.0	11.3
HX-ΔBB	0.087	0.167	0.124	0.121	0.37	0.250	0.026	<0.010	0.203	1.04
89.6	81	86	91	154	90	108	4.2	16.2	3.9	168
89-ΔBB	21.6	21.0	112	91	52	35	5.8	12.0	127	342
89-ΔBM	1.21	0.80	5.0	9.0	16.0	2.9	0.40	0.34	13.0	55
89-ΔMB	249	410	100	236	19.0	6.1	1.30	19.0	32	53

^a Cells were infected as described in Materials and Methods and maintained for 4 weeks.

^b Values are peak levels of p24^{agg} antigen (ng/ml) in the supernatant in replicate plates of macrophages from different donors in five independent experiments.

^c ND, not determined.

defects in each of these open reading frames, as well as HXB U3 long terminal repeat sequences (Table 2). Previous studies have shown that *vpr*, *vpu*, and *nef* each contribute to maximal HIV-1 replication in macrophages (2, 3, 27, 38), but whether this is responsible for the lower levels reached by HX-ΔKK in MDM compared with 89.6 or whether these levels result from additional efficiency factors present in the 3' genome but outside the *KpnI-KpnI* fragment is unclear. To test the reciprocal exchange, the identical region of HXB was inserted into 89.6, but this chimera was incapable of replication in either PBL or CEMX174 cells (data not shown), and thus it was not useful for this analysis of tropism. The reasons behind this phenomenon are currently under study.

Because the V3 region has been repeatedly identified for other strains as central to the macrophage-tropic phenotype, we tested specific reciprocal exchanges of a region that codes for 198 amino acids of *env* which includes V3, V4, and V5 without the involvement of any other genes. Surprisingly, replacement of this region of HXB with sequences derived from 89.6 in the chimera HX-ΔBB did not result in productive macrophage infection in any of the multiple replicate experiments (Fig. 2A and Table 1), even though this virus was fully replication competent in PBL and CEMX174 cells. Even more striking, the reciprocal exchange in which the V3, V4, and V5 regions of HXB were introduced into the background of 89.6 did not affect macrophage tropism, and the 89-ΔBB chimera produced antigen titers in MDM that were similar to those produced by wild-type 89.6 (Fig. 2B and Table 1). Taken together, these results indicate that in 89.6 the elements which confer the ability to replicate in macrophages lie within the

2.7-kb *env*-containing *KpnI-KpnI* fragment but do not involve V3 or V4/V5 and that the V3 through V5 regions of the non-macrophage-tropic HXB clone are competent to support replication in macrophages when placed in the background of strain 89.6.

Because several groups have emphasized the importance of interactions between domains of gp120 (1, 16, 17, 25), we generated chimeric viruses 89-ΔBM and 89-ΔMB. In 89-ΔBM, the V3 region alone of HXB was inserted into 89.6, while 89-ΔMB contained HXB sequences that included V4 and V5. 89-ΔMB replicated in MDM with kinetics similar to those of wild-type 89.6, confirming the finding that V4 and V5 are dispensable for macrophage tropism. Interestingly, substitution of only the V3-containing region in 89-ΔBM resulted in antigen titers that were generally lower than those for 89.6, 89-ΔBB, or 89-ΔMB (Table 1), despite similar replication patterns in PBL and CEMX174 cells. Thus, the V3 region of HXB, when placed into the 89.6 background, requires its cognate V4/V5 sequences for maximum efficiency in macrophages but not in PBL. Since the V3 region of HXB is fully competent to support replication in MDM when placed into the background of 89.6 in 89-ΔBB, these results suggest that there are interactions between V3 and V4/V5 that modulate the efficiency of replication in a cell-specific manner. Functional interactions between various gp120 domains have similarly been reported by others (1, 16, 17, 25).

Confirmation of chimeric viruses by restriction analysis. Each recombinant proviral clone was verified prior to transfection. However, since these results differed from those reported for several other isolates (23, 31, 35, 37, 39), we sought

TABLE 2. Summary of recombinant viruses

Virus	<i>env</i> region exchanged ^a	Other regions exchanged ^b	Auxiliary gene defect(s)	Replication in PBL ^c	Replication in MDM ^c
HXB			<i>vpr</i> , <i>vpu</i> , <i>nef</i>	++++	—
HX-Δ3'	All	3' <i>vpr</i> , <i>vpu</i> , <i>tat</i> , <i>rev</i> , <i>nef</i> , U3	<i>vpr</i>	++++	++++
HX-ΔKK	All except 5'	<i>tat-2</i> , <i>rev-2</i> , 5' <i>nef</i>	<i>vpr</i> , <i>vpu</i> , <i>nef</i>	++++	++
HX-ΔBB	V3, V4, V5			++++	—
89.6				++++	++++
89-ΔBB	V3, V4, V5			++++	++++
89-ΔBM	V3			++++	++
89-ΔMB	V4, V5			++++	++++

^a V indicates designated hypervariable regions of gp120.

^b *tat-2* and *rev-2* indicate the second exons of *tat* and *rev*, respectively.

^c Replication is designated as follows: + + + +, productive infection similar to that of 89.6 parental virus; + +, productive infection with reduced titers and/or delayed kinetics; —, nonproductive infection.

consensus	263	297	net (+) charge	M- tropic	syncytia- inducing
N. American	CTRPNNTKRKSIHI••GPGRAFYTGTGEEIIGDIRQAHC		5	--	--
M-tropic	-----••-----A-----		5	yes	no
non-M-tropic	-----Q--RR--••-----RQ-----		7	no	yes
strain					
HXB2	-----R-R-QR-----V-I-K*--NM-----		10	no	yes
89.6	-----RRLS-••-----ARRN-----		8	yes	yes
JRFL	-----••-----		5	yes	no
ADA	-----••-----		5	yes	no
Bal	-----••-----L-----		5	yes	no
YU-2	-----N-••-----L-----		4	yes	no
SF162	-----T-••-----A--D-----		4	yes	no

FIG. 3. Predicted amino acid sequences of the third hypervariable (V3) loop. Sequences of HXB2 and 89.6 are compared with a North American V3 consensus sequence (26) and reported macrophage (M)-tropic and non-macrophage-tropic consensus sequences (9). Below them are aligned sequences of other macrophage-tropic viruses for which genetic mapping has indicated that regions of the envelope which include V3 are critical determinants of macrophage tropism (23, 31, 35, 37, 39). The net positive charge of each sequence is shown on the right, along with each strain's phenotype. Dots indicate gaps; dashes indicate conserved amino acids. Syncytia-inducing refers to infection of PBL or permissive T-cell lines.

to ensure the chimeric nature of the infectious viruses utilized in these experiments. Primers spanning the restriction sites utilized for exchanges were employed for PCR amplification of proviral DNA generated from the viruses, which was then subjected to restriction mapping with enzymes specific for one or the other of the parental clones. In all cases the restriction pattern was that expected on the basis of the recombination site and the parental restriction maps, confirming the identities of the infectious viruses (data not shown).

Analysis of V3 loop sequences. Strain 89.6 has 92.5% overall nucleotide sequence homology with HXB2 within the 3' half of the genome, and in *env* the two clones display 91.6% homology at the nucleotide level and predicted amino acid homology of 90.4%. Because of the importance of the V3 loop region for the viral phenotype and our findings of V3-independent macrophage tropism for strain 89.6, we compared the predicted protein sequence of the V3 loop of 89.6 with those of other molecularly cloned strains for which macrophage tropism has been linked to this domain. As shown in Fig. 3, those viruses exhibit a remarkable degree of similarity in their V3 sequences, while strain 89.6 has a markedly divergent pattern. Notably, the V3 sequences of the other strains are all quite similar to the V3 consensus sequence previously described (26), while that of 89.6 is distinct. In addition, the other strains are all less charged, while 89.6 has a strong positive charge which is similar to that of HXB and is more characteristic of cytopathic isolates than macrophage-tropic noncytopathic strains (15). Like the macrophage-tropic isolates shown in Fig. 3, 89.6 belongs to the major North American clade B group (30). Comparison of genetic distances, however, did not suggest that the V3 sequence of 89.6 was more closely related to that of HXB than to those of the other macrophage-tropic viruses (data not shown).

DISCUSSION

In this study we genetically analyzed the macrophage-tropic HIV-1 variant 89.6 by using chimeras generated with the prototypic non-macrophage-tropic strain HXB2 and showed that in this pair of viruses the elements responsible for macrophage tropism in strain 89.6 are localized to a 2.7-kb *env*-containing region in the 3' end of the genome but that they exclude the V3 region. This is the first demonstration of molecular determinants of macrophage tropism in HIV-1 that are independent of the *env* V3 domain. Our results stand in marked contrast to

those of several previous studies which used other cloned macrophage-tropic strains and demonstrated that the V3 region was the principal determinant of macrophage tropism, although additional flanking *env* sequences were often required for maximal replication (23, 31, 35, 37, 39). Taken together, these results indicate that there are multiple alternative elements within the viral genome that can confer macrophage tropism.

In addition to the novel finding that the V3 region of 89.6 is not a determinant of macrophage tropism, these chimeras localized the region responsible for macrophage tropism in 89.6 to a fragment that contains essentially all of *env* along with the second exons of *tat* and *rev* and the 5' portion of *nef*. While *env* is most likely the element responsible for tropism of this virus, a contribution by *tat*, *rev*, or *nef* has not been excluded. Several additional recombinants in which other *env* fragments were exchanged between the clones were generated, but they were unsuitable for analysis of macrophage infection because of their failure to replicate permissively in lymphoid cells, the reasons for which are currently under study. Whether the determinants of macrophage tropism for 89.6 will localize to a discrete region instead of V3 or whether they will be more complex and dispersed throughout *env* as has been described for simian immunodeficiency virus (29) remains to be determined. Interestingly, when combined with data from previous studies mapping macrophage tropism in HIV-1, our results show a parallel to results with simian immunodeficiency virus, as both V3-dependent (21, 24) and V3-independent (4, 29) determinants of macrophage tropism have been described for that model. Furthermore, although this is the first description of macrophage tropism determinants in HIV-1 that exclude V3, several studies have identified elements in *env* domains independent of V3 that affect tropism for cell lines (7, 12).

The mechanisms of infection which differ between cell types and underlie differential host cell tropism are poorly understood. It has been proposed that the V3 loop may be a target for sequence-specific proteolytic cleavage that is involved in entry and that relative resistance to cleavage characterizes macrophage-tropic viruses (18, 23). Our results suggest that if such a mechanism is involved, it is more likely related to structural effects of complex interactions among *env* regions than to sequence-specific phenomena. Functional interactions between gp120 domains, including modulation of V3 structure and function by V1, V2, and other elements of gp120, have

been well described (16, 17, 36), and the gp120 structure required for macrophage infection may be supported by alternative determinants contributed by distinct regions of the envelope. Alternatively, it may be that there are multiple mechanisms, supported by distinct determinants, by which viruses can establish productive infection in macrophages. Such a possibility is supported by the fact that in some studies with both HIV and simian immunodeficiency virus macrophage tropism appeared to be determined by early events such as viral entry (31), while other reports pointed to later post-reverse transcription events (22, 28).

The macrophage-tropic strains for which previous genetic mapping identified V3-dependent determinants have V3 loop sequences which are extremely similar to one another and to the North American V3 consensus sequence (26), and they have a relatively less-charged amino acid composition. They are also biologically similar, as they are NSI in T cells. In contrast, 89.6 is highly SI in T cells, and it has a distinct V3 sequence that carries a strong positive charge characteristic of SI but not NSI isolates (9, 15). Since our results demonstrate that macrophage tropism in a naturally occurring isolate may be independent of V3, the marked homogeneity of V3 sequences among many macrophage-tropic viruses suggests that factors other than macrophage tropism per se may have played a role in their selection. Similarly, Chesebro et al. found that even though sequences derived from a panel of macrophage-tropic isolates were similar, many divergent patterns generated by mutagenesis in vitro were compatible with macrophage infection (9). Since macrophage-tropic variants appear to be the principal virus type present in individuals immediately after primary infection (40, 41), they may play a role in virus transmission or acquisition, during which time such selection may occur. In addition, they are the main type detected during the prolonged asymptomatic period (33), and they may be important in persistence and dissemination of the virus. V3 sequences are known to influence SI and NSI characteristics (5, 13), and it is possible that coselection for the NSI phenotype is responsible for the homogeneity among described macrophage-tropic V3 sequences. Alternatively, immune factors may play a role, since V3 is an important target for immune responses and both typical macrophage-tropic isolates and isolates with the "macrophage-tropic" V3 pattern appear to be resistant to antibody neutralization (6, 8). Interestingly, 89.6 was isolated from an individual with far-advanced immune deficiency, and thus it may have escaped from such selective factors.

In this study we found that a fragment including V3 derived from the non-macrophage-tropic clone HXB supported efficient macrophage infection when placed in the background of 89.6. This demonstrates that the proviral context in which a V3 sequence is analyzed can significantly influence its function. PCR-generated envelope sequences obtained directly from tissues are being used with increasing frequency to analyze virus populations (32, 40). Our results urge caution in using pure sequence information to biologically characterize viruses and suggest that phenotypic characterization of V3 sequences should be considered specific to the viral context in which they are placed.

The chimeric virus HX- Δ KK, which has nearly the entire *env* from 89.6, productively infected macrophages but with peak antigen titers that were approximately 10-fold lower than those for wild-type 89.6. HX- Δ KK has defects in its *vpr*, *vpu*, and *nef* open reading frames, which are derived from the parental HXB2 clone. We recently reported that mutagenesis of these accessory genes in the native 89.6 clone restricted replication in macrophages, although in that study the absence of all three

genes impaired replication in macrophages to a considerably greater degree (2). On the other hand, HX- Δ 3' possesses the entire 3' genome derived from 89.6 but has the *gag*, *pol*, *vif*, and truncated *vpr* open reading frames of HXB2. Interestingly, this virus was not restricted compared with 89.6, which contrasts with the results of our previous study, in which we found macrophage infection to be highly *vpr* dependent. This likely relates to differences in the viruses employed, as quantitatively different results have been reported for other studies of *vpr* function in macrophages (3, 19, 38). It was recently reported that *vpr* assists in nuclear migration of the preintegration complex in macrophages in a manner similar to that of the nuclear localization sequence of *gag* and that the effects of *vpr* differ in the context of different nuclear localization sequences (20), supporting the possibility that different viral backgrounds may influence the role played by *vpr* in macrophage infection.

Whether V3-independent determinants of tropism are common among other macrophage-tropic viruses is uncertain. The pattern identified in this analysis may represent a rare case which nevertheless can offer valuable insight into common structural elements and potential mechanisms involved in differential host cell tropism. On the other hand, it may reflect a common pattern, since the isolates so far subjected to genetic mapping are mainly derived from the North American/European clade B subtype and strains with widely divergent *env* sequences, particularly those from other clades, have yet to undergo this type of study. Our results emphasize the importance of genetic analysis using multiple isolates with divergent genetic and biological characteristics.

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