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Identification of the Envelope V3 Loop as the Primary Determinant of Cell Tropism in HIV-1

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Cells of the monocyte-macrophage lineage are targets for human immunodeficiency virus–1 (HIV-1) infection in vivo. However, many laboratory strains of HIV-1 that efficiently infect transformed T cell lines replicate poorly in macrophages. A 20–amino acid sequence from the macrophage-tropic BaL isolate of HIV-1 was sufficient to confer macrophage tropism on HTLV-IIIB, a T cell line–tropic isolate. This small sequence element is in the V3 loop, the envelope domain that is the principal neutralizing determinant of HIV-1. Thus, the V3 loop not only serves as a target of the host immune response but is also pivotal in determining HIV-1 tissue tropism.

ALTHOUGH THE $CD4^+$ LYMPHOcyte is the major target for HIV-1 replication in the peripheral blood compartment, cells of the monocyte-macrophage lineage represent the predominant HIV-1–infected cell type in most tissues, including the central nervous system (*1–3*). An HIV-1 infection of macrophages, although less cytopathic than an infection of T cells, compromises macrophage function and may underlie many of the pathogenic effects of HIV-1 infection in humans (*1–3*).

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Despite the importance of macrophages as a primary target for HIV-1 infection in vivo, many laboratory isolates of HIV-1 are unable to replicate in these cells (*3–8*).

The HIV-1 isolates can be divided into two major subgroups on the basis of their cellular host range in vitro (*2–9*). Macrophage–T cell (MT)–tropic isolates efficiently infect both macrophages and $CD4^+$ peripheral blood lymphocytes (PBLs) but are unable to replicate efficiently in many transformed cell lines of either T cell or monocytic origin. A second class of viruses, termed T cell (T)–tropic, replicates efficiently in both PBLs and transformed T cell lines but poorly in macrophages (*2–9*). The tropism of HIV-1 is determined early in the viral replication cycle, between binding of the virus to the cell surface and initiation of viral reverse transcription (*6, 7*) and is independent of the viral long terminal repeat (*8*) but dependent on sequences in the viral gp120 envelope protein (*5, 6*).

The HTLV-IIIB isolate of HIV-1 is T-tropic, whereas the BaL strain is MT-tropic (*3, 8*). To determine which sequences were important for in vitro tropism, we cloned and sequenced the envelope gene and flanking viral sequences of BaL with DNA derived from BaL-infected macrophages (*10*). We then constructed a series of chimeric HIV-1 proviruses by substitution of BaL-derived sequences into an HTLV-IIIB proviral clone (Fig. 1). These chimeric viruses were then tested for tropism by analysis of their replication competence in PBLs (*11*), primary monocyte-derived macrophages (*12*), and the transformed $CD4^+$ T cell lines H9 and CEM (Table 1).

Both the parental HTLV-IIIB clone (pIIIB) and the provirus with the complete BaL *env* gene (pBaL) replicated equivalently in primary PBLs, as we determined by measuring secreted p24 Gag protein (Table 1) or supernatant reverse transcriptase activity (*13*). As predicted (*3, 8*), the pIIIB provirus also replicated efficiently in both the H9 and CEM cell lines but not in macrophages. In contrast, the pBaL provirus replicated efficiently in macrophages but not in H9 or CEM cells (Table 1). The various chimeric proviral constructs, like the parental pIIIB and pBaL clones, all displayed comparable replication competence in PBLs.

In addition, all chimeric clones were either fully T-tropic or fully MT-tropic; that is, no intermediate or dual tropism was detected. As previously reported (*5, 6*), tropism was determined entirely by sequences located within gp120. A 20–amino acid

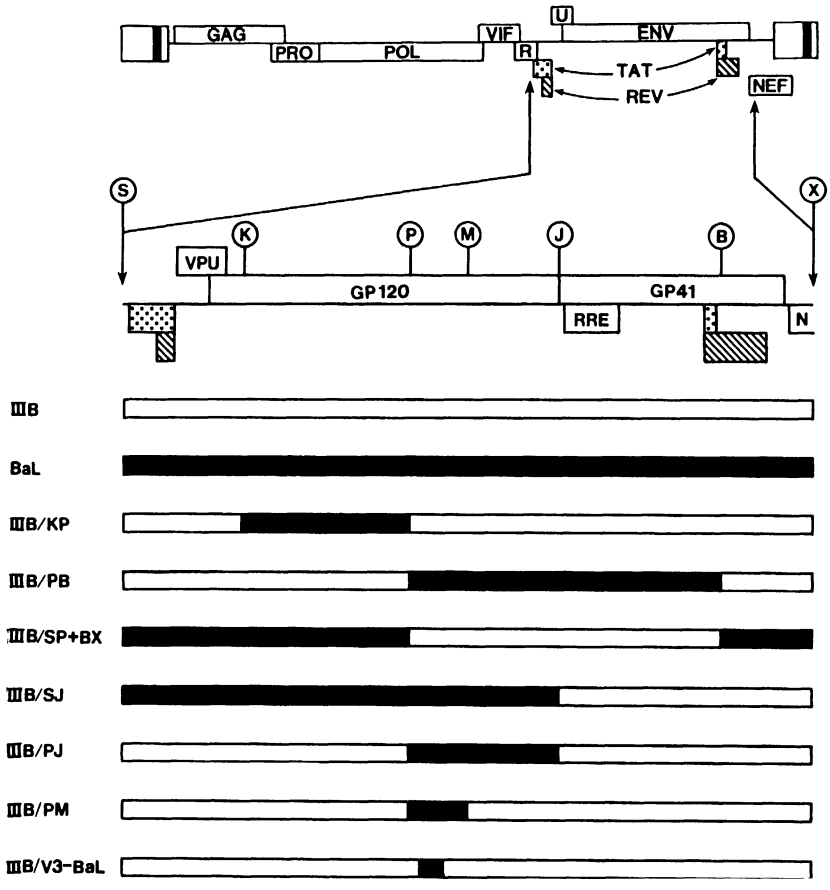
BaL-specific sequence that introduces only 11 amino acid changes into the pIIIB envelope protein was both necessary and sufficient to confer a fully MT-tropic phenotype. An HTLV-IIIB–derived provirus bearing this minimal substitution, termed IIIB/V3-BaL, was observed to replicate in macrophages at least as well as the pBaL proviral clone, yet could no longer infect T cell lines (Table 1). The introduced 20–amino acid BaL-specific sequence coincides with the core of the V3 loop of the HIV-1 envelope protein (Table 2), a discrete ~35–amino acid protein domain that forms the principal neutralizing determinant of HIV-1 (*14–16*). Our data demonstrate that the V3 loop is also a major determinant of HIV-1 cell tropism.

A 159–amino acid envelope sequence has been identified as a critical determinant of HIV-1 tropism (*5, 6*). This longer sequence extends 101 amino acids NH_2-terminal and 38 amino acids COOH-terminal to the 20–amino acid V3 loop sequence defined here, suggesting that the V3 loop may also be a major determinant of tropism in other viral isolates. These experimentally defined MT-tropic V3 loop sequences are all similar to each other and to the statistically prevalent or "consensus" V3 loop sequence defined by examination of 245 distinct isolates of HIV-1 (*16*) (Table 2). In contrast, T-tropic isolates appear to be characterized by statistically rare V3 loop sequences that are dissimilar to the consensus. It therefore appears possible that envelope V3 loop sequences may help predict the cell tropism of primary HIV-1 isolates.

MT-tropic isolates are the predominant HIV-1 class detected early after infection of humans, whereas T-tropic isolates become more prevalent as disease progresses (*9*). This progression may be involved in HIV-1 pathogenesis (*9*). Similarly, the V3 loop sequence is also subject to rapid change both within and between different human hosts (*15, 16*). This evolution has been ascribed to the immunological selection of virus-bearing variations in the V3 loop sequence that permit escape from V3 loop–specific neutralizing antibodies (*15*). However, our data suggest that V3 loop evolution may also reflect the selection of T-tropic variants with nonconsensus V3 loop sequences during the later stages of HIV-1–induced disease. Therefore, vaccines intended to protect against challenge by the MT-tropic isolates prevalent during the early stages of HIV-1 infection should perhaps be designed to elicit an immune response specific for V3 loop sequences similar to the consensus.

The mechanism by which the V3 loop influences HIV-1 cell tropism remains unclear. The V3 loop probably does not interact with a primary cell surface receptor that is distinct from CD4. CD4 binding is critical for infection of both macrophages and T cells (*17*), and a defective or occluded V3 loop does not affect the ability of gp120 to bind cell surface CD4 (*14*). Thus, the V3 loop is likely to be involved in a step immediately subsequent to the initial gp120-CD4 binding event, which results in the activation of the fusogenic potential embodied in the hydrophobic NH_2-terminus of the gp41 component of the envelope (*18*).

An activation step would preclude the premature fusion of gp41 with cell membranes encountered during the intracellular posttranslational processing of envelope and would also prevent the random fusion of virions with $CD4^-$ cells unable to support viral replication, such as reticulocytes (*18, 19*). In some enveloped animal viruses, activation of fusion occurs subsequent to a low pH-induced conformational change in the viral membrane protein after endocytosis of the virion (*19, 20*). However, fusion of HIV-1 with $CD4^+$ cells requires neither exposure to low pH nor internalization of the CD4 receptor (*20, 21*). Alternatively, envelope fusion in HIV-1 might require a specific proteolytic activation step, as has been suggested for a number of enveloped viruses, including the retrovirus murine leu-



g. 1. Structure of chimeric HIV-1 proviral clones. The HXB-3 proviral clone is full length and plication-competent and is derived from the HTLV-IIIB isolate of HIV-1 (*26*). The HXB-3 provirus esent in the pIIIB plasmid is similar to the published clone except that the single base pair frame-shift mutation present in the *vpr* gene of HXB-3 has been repaired (*27*). In the majority of cases, BaL sequences (in black) were substituted into the pIIIB clone (in white) after digestion at the indicated restriction enzyme sites. However, some proviral chimeras were constructed with polymerase chain reaction primers specific for the gp120-gp41 junction or for the V3 loop region, in combination with flanking primers homologous to sequences adjacent to the unique viral Kpn I and Bam HI sites (*10*). In the case of pIIIB/V3-BaL, the primers used were 5′-GGGTCCTATATGTATACTTTTTCTTG-TATTGTTGTTGGG-3′ and 5′-AAAAAGTATACATATAGGACCCGGGAGAGCATTATATACA-ACAGGAGAAATAATAGGAGATATAAG-3′. These primers permitted the construction of a substitution mutant, pIIIB/V3-BaL, that is identical to the pIIIB parent except for a region of 20 amino acids in the core of the V3 loop sequence (Table 2). The origin of the various chimeric proviral clones is indicated in the plasmid name given at left (for example, IIIB/KP contains a BaL-derived Kpn I to Pvu II fragment) and was confirmed by dideoxynucleotide sequence analysis. K, Kpn I; P, Pvu II; M, Mst II; J, gp120-gp41 junction; B, Bam HI; RRE, Rev Response Element; R, Vpr; U, Vpu; N, Nef; S, Sal I; X, Xho I.

Table 1. Cell tropism of chimeric HIV-1 proviral clones as measured by p24 Gag expression in picograms per milliliter. To assess tropism, we transfected HIV-1 proviral clones (*25*) into cultures of the monkey cell line COS that were in 35-mm plates. At 3 days after transfection, the supernatant media was replaced by 2 ml of RPMI medium with PHA-stimulated PBLs (2×10^6) (*11*). Activated PBLs were cultivated with the transfected COS cells for 3 days, aspirated, washed, and maintained in expanded culture for four more days. On day 7 (d7) after infection, supernates (10 ml) were removed from the PBLs and filtered through a 0.45 μM filter, and p24 was assayed by enzyme-linked immunosorbent assay (DuPont Biotechnology Systems). These values are given in the column marked "PBL." The p24 levels were then standardized by dilution to ~200 pg/ml (experiment 1) or ~300 pg/ml (experiment 2), and we used 500 μl of each virus supernatant to infect adherent monocyte-derived macrophages (MØ) (*12*), 5×10^5 H9 cells, or 5×10^5 CEM cells. Supernatant media were replaced twice per week and monitored for p24 expression levels. The data given are for 14 days (d14) after infection, but similar results were also routinely obtained at days 7 and 21. No p24 expression was detected in negative control cultures.

Chimera	Experiment 1				Experiment 2		
	PBL d7	MØ d14	H9 d14	CEM d14	PBL d7	MØ d14	H9 d14
IIIB	306	<3	1368	474	572	11	138
BaL	341	627	<3	<3	296	298	5
IIIB/KP	368	<3	927	442	696	5	298
IIIB/PB	272	1161	<3	<3	322	301	5
IIIB/SP+BX	356	<3	505	629	585	3	344
IIIB/SJ	248	694	<3	<3	564	259	<3
IIIB/PJ	347	1250	<3	<3	340	252	3
IIIB/PM	366	1426	<3	<3	448	616	<3
IIIB/V3-BaL	291	2172	<3	<3	364	709	<3

Table 2. Comparison of V3 loop sequences of HIV-1 isolates of known tropism. The ~35-amino acid V3 loop is a discrete envelope protein domain defined by two invariant, disulfide-bonded cysteine residues (*15*). A statistically prevalent or consensus V3 loop sequence has been defined (*16*) and this is identical to the V3 loop of the MT-tropic isolate JR-FL (*6*). Modification of the HTLV-IIIB V3 loop sequence to match that of the BaL isolate, by exchange of the boxed amino acids, is sufficient to confer an MT-tropic phenotype. This mutation introduces 11 amino acid changes into the envelope protein of HTLV-IIIB. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. Each dash indicates identity with the BaL sequence; each dot, a deletion.

Clone	Sequence	Tropism
SF-2	---------- --Y-••------FH---R----- -K---	T
NL4-3	---------- --R-QR-----FV-I-K•--NM -----	T
HTLV-IIIB	----------K-R-QR-----FV-I-K•--NM-----	T
BaL	CTRPNNNTRKSIHI••GPGRALYTTGEIIGDIRQAHC	MT
JR-FL (consensus)	---------- ----••------F---------- -----	MT
SF162	---------- --T-••------F-A--D----- -----	MT

kemia virus (*20, 22, 23*). HIV-1 tropism would then reflect the availability of cell surface or lysosomal proteases capable of cleaving specific sites within different viral V3 loops. Evidence suggesting that the V3 loop is a target for sequence-specific proteases has been presented (*24*), and the availability of appropriate cellular proteases is known to affect tissue tropism in some other viral species (*23*). If the sequence-specific cleavage of V3 is indeed critical for HIV-1 infection, then this site might well provide a novel and attractive target for chemotherapeutic intervention in HIV-1–induced disease.

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The *ras* Oncoprotein and M-Phase Activity

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The endogenous *mos* proto-oncogene product (Mos) is required for meiotic maturation. In *Xenopus* oocytes, the *ras* oncogene product (Ras) can induce meiotic maturation and high levels of M-phase–promoting factor (MPF) independent of endogenous Mos, indicating that a parallel pathway to metaphase exists. In addition, Ras, like Mos and cytostatic factor, can arrest *Xenopus* embryonic cell cleavage in mitosis and maintain high levels of MPF. Thus, in the *Xenopus* oocyte and embryo systems Ras functions in the M phase of the cell cycle. The embryonic cleavage arrest assay is a rapid and sensitive test for Ras function.

IN THE *XENOPUS LAEVIS* SYSTEM, FULLY grown oocytes are arrested in prophase of the first meiotic division. Progesterone releases this arrest, resulting in activation of MPF, germinal vesicle breakdown (GVBD), completion of meiosis I, and production of an unfertilized egg arrested at metaphase II of meiosis (*1*). MPF is composed of the *Xenopus* homolog of the cell cycle regulator $p34^{cdc2}$ and cyclin (*2*) and is present at high levels in unfertilized eggs (*1*). Cytostatic factor (CSF) is also found in unfertilized eggs and is believed to be responsible for the arrest of maturation at metaphase II of meiosis (*1, 3*). Mos has been shown to be an active component of CSF (*4*), and introduction of CSF or Mos into the blastomeres of rapidly cleaving embryos arrests cleavage at metaphase of mitosis (*1, 3, 4*). This arrest by CSF or Mos, at a major cell cycle control point (*5*), results from the stabilization of high levels of MPF (*3, 4, 6,* 7).

The unrestricted proliferation of cells transformed by oncogenes provides a strong argument that proto-oncogenes normally function in the regulation of the cell cycle (*8*). Research emphasis has been directed toward understanding how oncogenes alter the regulation of signal transduction events in the G_0 to G_1 phase of the cell cycle (*9*). The discovery that Mos functions during M phase (*4, 10*) led us to propose that the transforming activity of the Mos in somatic cells is due to the expression of its M-phase activity during interphase (*4, 10, 11*). A similar hypothesis has been presented for the *src*-transforming activity (*12*), and this may be a more general mechanism for how certain oncogenes induce morphological transformation (*4, 10, 11*).

Ras, the transforming guanosine triphosphate (GTP)–binding protein (*13*), and Mos induce progesterone-independent meiotic maturation in *Xenopus* oocytes (*11, 14–17*) (Table 1). We tested Ras in this assay by injecting either Ras^{Lys12} or $H\text{-}Ras^{Val12}$ RNA. Injected oocytes were subsequently examined for GVBD and MPF activity (*18*). Cytosolic extracts prepared from oocytes induced to mature with these products were positive for MPF, indicating that the oocytes were arrested in metaphase (Table 1). In addition, these analyses confirm that Ras (*19*), like Mos, can sustain high levels of MPF after GVBD (Table 1).

In fully grown *Xenopus* oocytes, antisense oligodeoxyribonucleotides destabilize the *mos* maternal mRNA and block progesterone-induced meiotic maturation (*10, 15*). To test whether Ras could induce meiotic maturation in the absence of progesterone and endogenous *mos* mRNA, we injected *mos*-specific antisense or sense oligodeoxyribonucleotides (*10*) into oocytes 3.5 to 4 hours before injecting the test material and subsequently examined them for GVBD and MPF activity (Table 1). GVBD occurred frequently in Mos-negative oocytes injected with Ras (60%), and extracts prepared from oocytes that displayed GVBD were positive for MPF activity (Table 1). Barrett and co-workers have shown that Mos depletion inhibits Ras-induced maturation (*15*). Allende and co-workers reported that Ras can induce GVBD in cycloheximide-treated oocytes (*16*), and Barrett and co-workers also observed this on occasion (*15*). These latter results are more consistent with our data because Mos is not synthesized in oocytes in the presence of cycloheximide (*11, 20*). Moreover, Ras-induced oocyte maturation appears to be Mos-dependent in less mature Dumont stage V (*21*) oocytes, but not in fully grown stage VI oocytes (*22*), presumably because of metabolic changes during oogenesis.

Because Ras induces meiotic maturation and high levels of MPF in oocytes, we tested whether it influences M-phase events in cleaving embryos, where the cell cycle consists essentially of S and M phases. Ras efficiently arrested embryonic cleavage when one blastomere of each two-cell embryo was injected with either oncogenic Ras protein or RNA (Figs. 1 and 2). This cleavage arrest mimics the arrest caused by CSF or Mos (*4*). Moreover, as little as 1 to 2 ng of Ras could induce the cleavage arrest, which was observable within a few hours (Fig. 2).

Although transforming Ras induced the cessation of embryonic cleavage, both normal and nontransforming mutant forms of Ras had no observable effect on cleavage, even when introduced at concentrations approximately ten times the minimum effective dose for the transforming Ras. Thus, 15 ng of either normal Ras or $Ras^{Lys12Ser186}$, a protein that cannot associate with the plas-

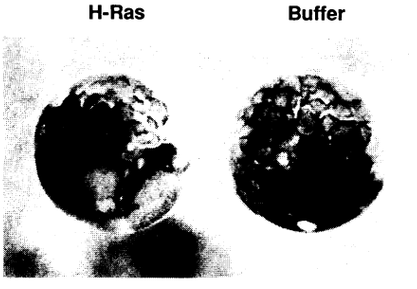

Fig. 1. Morphology of embryos injected with $H\text{-}Ras^{Val12}$ RNA. Animal-pole view of embryos injected with either capped $H\text{-}Ras^{Val12}$ RNA (*18*) or buffer. The RNA or buffer was microinjected into one blastomere (bottom half) of a two-cell embryo and examined several hours later.

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