Identification of Cryptic MHC I–restricted Epitopes Encoded by HIV-1 Alternative Reading Frames

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Abstract

Human immunodeficiency virus (HIV) 1 major histocompatibility complex (MHC) I–restricted epitopes are widely believed to be derived from viral proteins encoded by primary open reading frames. However, the HIV-1 genome contains alternative reading frames (ARFs) potentially encoding small polypeptides. We have identified a panel of epitopes encoded by ARFs within the *gag*, *pol*, and *env* genes. The corresponding epitopic peptides were immunogenic in mice humanized for MHC-I molecules. In addition, cytotoxic T lymphocytes recognizing these epitopes were found in HIV-infected patients. These results reveal the existence of atypical mechanisms of HIV-1 epitope generation. They indicate that the repertoire of epitopes recognized by the cellular anti–HIV-1 immune response is broader than initially thought. This should be taken into account when designing vaccine strategies aimed at activating these responses.

Key words: HLA-B7 • CTL • transgenic mice • KO mice

Introduction

Cellular immunity plays an important role in the pathogenesis of HIV-1 infection. HIV-1–specific CD8+ CTLs are involved both in the initial decrease of viremia during acute infection and in limiting viral replication in chronic stages of the disease (1–5). In vitro studies have demonstrated inhibition of HIV-1 replication by CTLs, by both lytic and nonlytic mechanisms (6, 7). However, despite the strong antiviral effects of CTLs, most infected individuals control viremia poorly in the absence of antiviral treatment. Several cellular and viral factors likely contribute to the failure of the immune system to control HIV-1 infection (7). Moreover, there is no clear association between viral loads and the breadth or magnitude of CTL responses in humans (8–10), suggesting either a lack of correlation between those two parameters, or technical limitations in the assays used to measure specific cellular immunity.

Thus, it is of interest to precisely document the repertoire of viral epitopes recognized by HIV-1–specific CTLs. Responses against all HIV-1 proteins have been reported, with the strongest ones clustering in the more conserved regions of Gag, Pol, and Nef. The viral proteins Vpu, Tat, Rev, Vif, Vpr, and Env seemed initially less frequently targeted (8, 9, 11–15). Recent studies performed using autologous virus rather than reference strains (8, 9, 14, 16) demonstrated that responses against variable regions of regulatory and accessory viral proteins are more common than previously reported. Notably, most, if not all, of the HIV-1 epitope studies have only assessed proteins encoded by primary open reading frames (ORFs) of the viral genome.

Nontraditional epitopes, derived from alternative reading frames (ARFs) have been reported for influenza virus (17, 18); melanoma, gut, and renal cancers (19–22); and an autoimmune disease (23). This is also the case for murine AIDS, which is caused by a mixture of retroviruses called LP-BM5. CTLs directed against a peptide encoded by a +2 ORF of a LP-BM5 *gag* gene are generated during the course of natural infection (24). Various transcriptional and

translational mechanisms may account for ARF expression and nontraditional epitope generation. These include cryptic promoter activation, reinitiation of translation at downstream start codons, and ribosomal frameshifting (25).

Based on these observations, we asked whether nontraditional CTL epitopes may be generated during HIV-1 infection. First, we identified ARFs within the HIV-1 genome that potentially encode small polypeptides (ARF polypeptides [ARFPs]). Using an algorithm for predicting HLA-B7–restricted epitopes, we selected candidate peptides encoded by these ARFPs. Their immunogenicity was evaluated using HLA-B7 transgenic, H-2K^{b}D^b double knockout mice (HLA-B7^{mα3} mice; reference 26). One such ARFP-derived peptide (Q9VF), encoded by a region overlapping the *gag* gene, was processed and presented by HIV-infected cells in vitro. In addition, CTLs recognizing this epitope were found in HIV$^+$ patients. Five additional ARFP-derived epitopes derived from *pol* and *env* ARFs were also studied. Altogether, these results reveal the existence of nontraditional mechanisms for HIV-1 epitope generation. They indicate that the extent of the cellular immune response directed against HIV-1 may be broader than initially thought.

Materials and Methods

Study Population.   PBMCs were obtained from eight HLA-B7$^+$, hepatitis C virus (HCV)$^-$, CMV$^+$, and HIV-infected individuals (patients nos. 1–8) from the Hôpital Européen Georges Pompidou with informed consent. Patients were asymptomatic and undergoing antiretroviral therapy (see Table I for patients' characteristics). Seven HIV$^-$ HLA-B7$^+$ (nos. 9a–9g) and one HIV-infected HLA-B7$^-$ (no. 10) individuals were also included in the study. HLA-B7 phenotyping was performed using anti–HLA-B7 ME.1 antibody (27). This phenotyping was confirmed by PCR amplification and sequencing of the genomic DNA, using a previously described method (28).

Cell Culture.   Ficoll-purified human PBMCs were either immediately used in antigen presentation assays or grown in 96 well-plates in RPMI 1640, supplemented with 10% human serum (Institut Jacques Boy) and human IL-2 at 20 IU/ml (Roche).

Mouse CTL lines were derived from splenocytes of immunized HLA-B7^{mα3} transgenic mice (26). In brief, these mice express a chimeric HLA-B0702 heavy chain with a murine α3 domain, and their H-2K^b and H-2D^b class I-a genes have been inactivated. Cells were cultured in RPMI 1640 supplemented with 10% FBS (Dutscher) and stimulated every 10 d with peptide-pulsed syngenic LPS lymphoblasts.

T2 (29), RMA (26), and T1 (174xCEM, CCR5$^+$ LTR-GFP$^+$) cells were stably transfected with the HLA-B7^{mα3} construct, yielding respectively T2-B7, RMA-B7, and T1-B7 cells, and were used as antigen-presenting cells.

Viruses and Infections.   HIV$_{LAI/stop}$ was generated by introducing in HIV$_{LAI}$ provirus a stop codon at position 3 in the ARFP amino acid sequence without affecting the primary Gag amino acid sequence, using Quickchange XL Site-directed Mutagenesis kit (Stratagene). HIV$_{LAI}$ and HIV$_{LAI/stop}$ were produced by transfecting the corresponding proviruses into 293T cells (30). 5 × 10^6 Jurkat and T1-B7 cells were infected with 500 ng of p24 for 3 h in culture medium containing 20 mM Hepes, pH 7.4, and 20

μg/ml DEAE-dextran. Culture supernatants were collected every 2–3 d and p24 content was measured by ELISA. After 5 d, infected T1-B7 cells were used as antigen-presenting cells in ELISPOT assays. At this time, 50% of the cells were positive for intracellular p24.

Mouse CTL Induction.   Peptide affinity for HLA-B0702 and immunogenicity assays were performed as described previously (26). In brief, HLA-B0702 binding of each peptide was assessed in a FACS®-based assay measuring the stabilization of empty HLA-B0702 molecules at the surface of 10^5 T2-B7 cells, and compared with the reference R10TV binding (CMV pp65 $_{265}$RPHERNGFTV$_{274}$; reference 31) as follows: the lower the relative affinity, the stronger the binding. Groups of six mice were immunized with single experimental peptides (50 μg/mouse) plus the helper peptide T13L (HBV-derived mouse IA^b MHC class II epitope), and spleen cells were restimulated in vitro (5 μg/ml of peptide). 6 d later, cytolytic activity was tested in a 4-h ^{51}Cr release assay using peptide-pulsed RMA-B7 target cells. Specific lysis was calculated as follows: (experimental − spontaneous release) (total − spontaneous release) × 100.

Mouse CTL Recognition of Infected T1 Cells.   80–2,000 mouse CTLs were stimulated by 100,000 infected T1-B7$^+$ cells, and IFN-γ release was detected by ELISPOT assays. Two mouse CTL lines specific for the HLA-B0702–restricted, known Nef-derived epitope F10LR (Nef$_{68}$FPVTPQVPLR$_{77}$; reference 32) or for the CMV-derived epitope R10TV were used as controls.

Human CTL Activity Assays.   Ex vivo peptide stimulation of human PBMCs was performed using HCV-derived HLA-B7–restricted epitope G9AT (GPRLGVRAT; reference 33) as negative control, phytohemagglutinin (Sigma-Aldrich) and CMV-derived epitope T10AM (pp65$_{417}$TPRVTGGGAM$_{426}$; reference 31) as positive controls, and known HIV-1–derived epitopes described in the Los Alamos Database as follows (http://www.hiv.lanl.gov/content/hiv-db/mainpage.html): S9WV, p24$_{16}$SPRTLNAWV$_{24}$; T9ML, p24$_{48}$TPQDLNTML$_{56}$; Y10LF, p6$_{36}$YPLASLRSLF$_{45}$; R10SI, gp160$_{298}$RPNNNTRKSI$_{307}$; I9GL, gp160$_{843}$IPRRIRQGL$_{851}$; F10LR, T10PL, Nef$_{128}$TPGPGVRYPL$_{137}$; R9AL, Nef$_{77}$RPMTYKAAL$_{85}$; and F9GL, Vpr$_{34}$FPRIWLHGL$_{42}$. For ELISPOT assay, 2 × 10^5 PBMCs were incubated for 16 h at 37°C with individual peptides (5 × 10^{-6} M), and IFN-γ release was measured as described previously (34). For cytofluorimetry analysis, PBMCs were plated for 2 h in V-bottom 96-well plates (600,000 cells/well) in IL-2–containing medium (5 IU/ml) and loaded with peptides at 5 μg/ml. 10 μg/ml of brefeldin A (Sigma-Aldrich) was added for 6 h, and intracellular IFN-γ production was measured as described previously (35) using the following mAbs: CD3-biotin, CD8-APC antibody, CD4-PerCP, streptavidin-PE, and anti–IFN-γ–FITC (BD Biosciences). Cells were analyzed with a FACS-Calibur™ cytometer (BD Biosciences). Percentage of IFN-γ–producing cells was obtained by gating on CD3$^+$ CD4$^+$ and CD3$^+$ CD8$^+$ cells. For ^{51}Cr release assay, 10^6 PBMCs were loaded with the relevant peptides at 5 μg/ml and cultured in 48-well plates. On day 3, 25 ng/ml recombinant human IL-7 (Sanofi-Synthelabo) was added. CTL lines were restimulated twice (at 10-d intervals) with peptide-pulsed γ-irradiated autologous PHA-blasts (50 Gy) in IL-2–containing medium. Cytolytic assays were performed as aforementioned using T2-B7 cells as targets. G9AT peptide was used as a negative control.

Statistical Analysis.   The nonparametrical Mann-Whitney test was performed using Abacus Concepts, Stat View statistic software. Differences were considered statistically significant when P < 0.05.

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783 -   AGAACTTᵀAAATGCATGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTGATACCCATGTTT
Gag     R  T  L  N  A  W  V  K  V  V  E  E  K  A  F  S  P  E  V  I  P  M  F
Gag-ARFP          M  G  K  S  S  R  R  E  G  F  Q  P  R  S  D  T  H  V  F

852 -   TCAGCATTᵀATCGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGACATCAA
GAG     S  A  L  S  E  G  A  T  P  Q  D  L  N  T  M  L  N  T  V  G  G  H  Q
Gag-ARFP  S  I  I  R  R  S  H  P  T  R  F  K  H  H  A  K  H  S  G  G  T  S  S

921 -   GCAGCCAᵀGCAAATGTTAAAAGAGACCATCAATGA
GAG     A  A  M  Q  M  L  K  E  T  I  N  E
GAG-ARFP  S  H  A  N  V  K  R  D  H  Q  *
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B
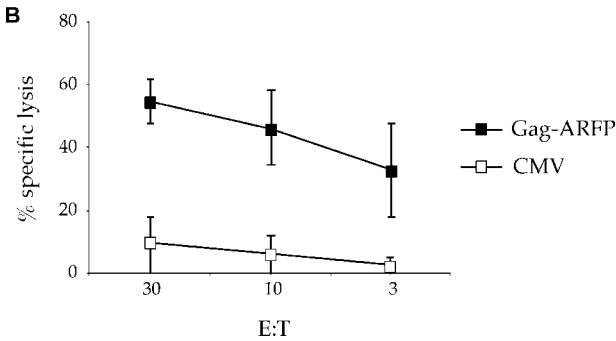
Figure 1. Identification of an antigenic epitope (Q9VF) derived from a nontraditional HIV-1 reading frame. (A) Nucleotide and amino acid sequences of a potential alternative reading frame (ARF) protein encoded within the *gag* gene. Start and stop codons are in bold, and the Kozak sequence is underlined. Nucleotide numbering is according to HIV$_{LAI}$ sequence. The predicted amino acid sequence of the corresponding polypeptide (Gag-ARFP) is shown, and the HLA-B7 potential epitope Q9VF is underlined (bold). (B) Cytotoxic activity of splenocytes from six HLA-B7 humanized mice immunized with Q9VF synthetic peptide. Splenocytes were tested using RMA-B7 target cells pulsed with the Q9VF peptide or with a nonrelevant CMV peptide (R10TV) as a control. Data are mean ± SD of six mice and are representative of three independent experiments. Cytolytic responses were considered positive when specific lysis was >10% at an effector/target (E/T) ratio of 30:1.

Results

Identification of Q9VF, a Potential MHC-I–restricted Epitope Derived from an HIV-1 ARF. In addition to the ORFs encoding the 10 major structural and regulatory viral proteins, the HIV-1 genome contains multiple ARFs that potentially encode small polypeptides (36). We asked whether immunogenic epitopes may be generated from these polypeptides. Polypeptides derived from ARFs that begin with an ATG codon and that present Kozak motifs (37) were analyzed using the SYFPEITHI algorithm (38) to predict which ones might be presented by HLA-B0702. We first identified a +2 ORF within the *gag* gene encoding a potential 52–amino acid polypeptide, which we named "Gag-ARFP" for Gag-ARF–encoded polypeptide (Fig. 1 A). Peptide Q9VF (Fig. 1 A, QPRSDTHVF), derived from Gag-ARFP, stably binds to the HLA-B0702 (HLA-B7) molecule with a relative affinity equivalent to the strong

A

B
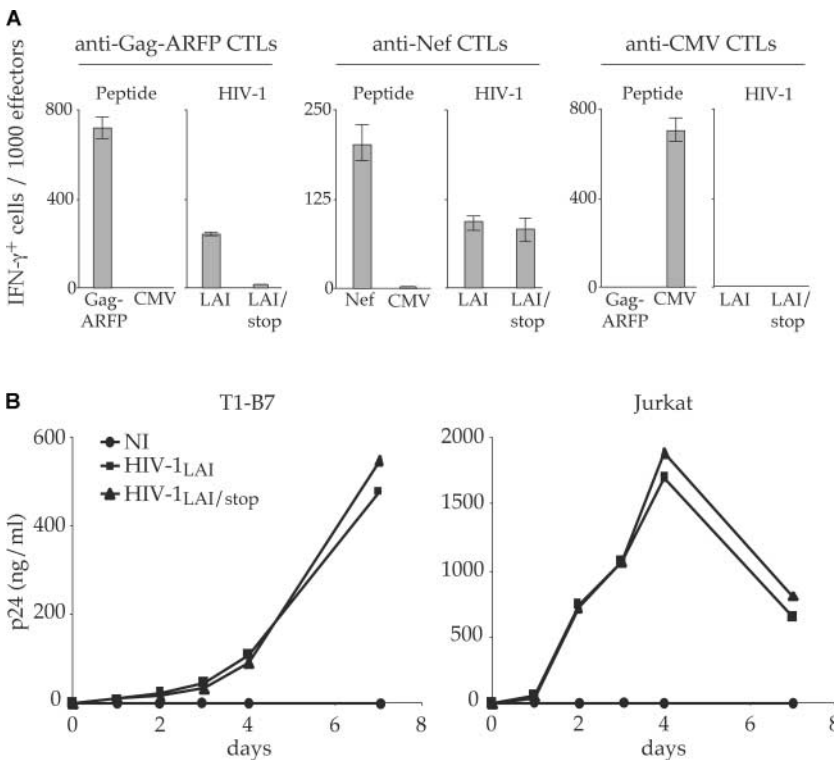
Figure 2. The Q9VF epitope from Gag-ARFP is presented by HIV-infected cells. (A) IFN-γ ELISPOT assay was performed using T1-B7 cells infected with HIV$_{LAI}$ or HIV$_{LAI/stop}$ or incubated with the indicated synthetic peptide as stimulators. Effector CTLs were splenocytes from HLA-B7 mice immunized with synthetic peptides corresponding to Gag-ARFP (Q9VF), HIV-1 Nef (F10LR), and CMV (R10TV) epitopes. Data are mean ± SD of triplicates for IFN-γ–producing cells per 1,000 effectors and are representative of three independent experiments. (B) Gag-ARFP is not required for HIV replication in T cell lines. The replication kinetics of HIV$_{LAI}$ and HIV$_{LAI/stop}$ were measured in two human cell lines (T1-B7 and Jurkat) by measuring p24 production in the supernatants at the indicated days after infection. HIV$_{LAI/Stop}$ was derived from HIV$_{LAI}$ by inserting a stop codon three nucleotides after the start codon of the sequence encoding for Gag-ARFP. T1-B7 cells were derived from T1 cells and express the HLA-B7^{mα3} protein. NI, noninfected cells.

binding of reference CMV peptide R10TV (not depicted). We found that transgenic mice expressing a human HLA-B7 protein, but deficient in mouse MHC class I molecules (HLA-B7$^{m\alpha3}$ mice) (26), produced a strong CTL response against peptide-pulsed targets after immunization with

Q9VF (Fig. 1 B). Thus, Q9VF is a CTL epitope presented by HLA-B7.

Q9VF Epitope Is Generated during HIV-1 Infection. We asked whether Q9VF is processed and presented during HIV-1 infection. To this end, we derived a human cell line that
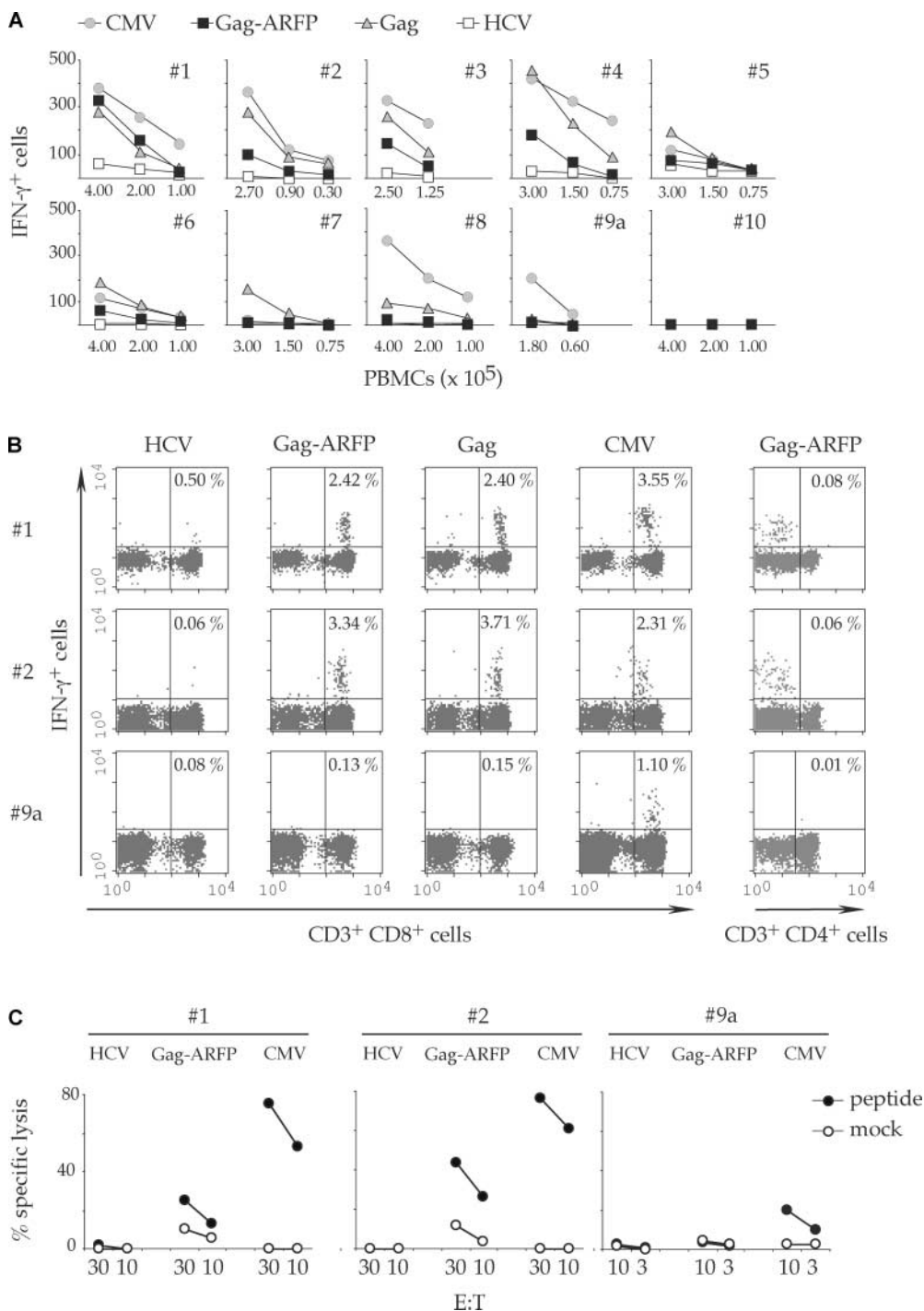
Figure 3. CTL responses to Q9VF in HIV-infected individuals. (A) IFN-γ ELISPOT assay was performed with PBMCs from eight HIV-infected HLA-B7$^+$ patients (nos. 1–8). As controls, PBMCs from seven HIV$^-$ HLA-B7$^+$ individuals (nos. 9a–9g) and one HIV$^+$ HLA-B7$^-$ (no. 10) individual were similarly analyzed. Responses against subject no. 9a are represented. The test was performed with the indicated concentrations of PBMCs and incubated with either CMV (T10AM), Gag-ARFP (Q9VF), Gag (Y10LF), or HCV (G9AT) peptides (at 5 μg/ml). Data are means of duplicates. Responses were considered positive when IFN-γ production was >50 spots/10^6 PBMCs and exceeded at least twofold background levels (measured with the HCV peptide). Similar results were obtained with Q9VF for individual nos. 9a–9g (not depicted). (B) IFN-γ intracellular labeling of PBMCs from HIV$^+$ HLA-B7$^+$ patients (nos. 1 and 2) and from an HIV$^-$ HLA-B7$^+$ individual (no. 9a). Cells were incubated with the indicated peptides, stained for CD3, CD4, CD8, and intracellular IFN-γ expression and analyzed by flow cytometry. The indicated percent corresponds to the proportion of IFN-γ^+ cells within CD3$^+$CD8$^+$ or CD3$^+$CD4$^+$ populations. Data are representative of three independent experiments. (C) Cytotoxic activity of PBMCs from two HIV-infected HLA-B7$^+$ patients (nos. 1 and 2) and one HIV$^-$ HLA-B7$^+$ individual (no. 9a). ^{51}Cr release assay was performed after two rounds of stimulation of PBMCs with the indicated peptides. Specific lysis was measured by using as targets T2-B7 cells pulsed with the corresponding peptides (closed circles) or with HCV G9AT peptide (open circles) as a control. Data are mean of duplicates at the indicated effector/target (E/T) ratio, and are representative of two independent experiments. Cytolytic responses were considered positive when specific lysis was >10% at an effector/target ratio of 30:1.

stably expresses the HLA-B7$^{m\alpha3}$ protein (T1-B7). These cells were infected with HIV$_{LAI}$ and used 5 d later as targets in an IFN-γ ELISPOT assay. At this time, ~50% of the cells were productively infected, as assessed by intracellular Gag p24 measurement (unpublished data). Effectors were murine CTL lines obtained by immunization of HLA-B7$^{m\alpha3}$ mice with either R10TV (a CMV peptide), F10LR (a classical HLA-B7–restricted HIV-1 Nef peptide), or Q9VF. CTLs recognizing HIV-1 Nef or Gag-ARFP–derived peptides were activated after coculture with infected targets (Fig. 2 A). As expected, the CMV peptide was not presented by HIV-infected T1-B7 cells.

To document the role of Gag-ARFP in viral replication and Q9VF antigen presentation, we derived a mutant virus (HIV-1$_{LAI/stop}$) in which the expression of Gag-ARF was abrogated by introducing a stop codon three nucleotides after the start codon without affecting the *gag* reading frame. Similar viral production and infectivity were observed with HIV-1$_{LAI}$ and HIV-1$_{LAI/stop}$ in T1-B7 and in Jurkat cells (Fig. 2 B) or in PBMCs infected with a lower viral inoculum (unpublished data). Altogether, this indicates that Gag-ARFP is not required for viral replication in these cells. Importantly, no significant IFN-γ production was observed in an ELISPOT assay using Q9VF-restricted mouse CTLs as effectors and HIV-1$_{LAI/stop}$–infected cells as targets (Fig. 2 A). In contrast, F10LR Nef-restricted CTLs efficiently recognized both HIV-1$_{LAI}$ and HIV-1$_{LAI/stop}$–infected cells (Fig. 2 A). Parental T1 cells lacking HLA-B7$^{m\alpha3}$ and infected with HIV$_{LAI}$ were recognized by neither Gag-ARFP nor Nef CTLs, indicating that antigen presentation

is appropriately MHC-I–restricted (unpublished data). Altogether, these experiments indicate that, whereas Gag-ARFP is not required for viral replication in T cell lines, the Q9VF epitope is generated in HIV-1–infected cells and is presented by MHC-I molecules, thus allowing activation of specific CTLs.

HIV-1–infected Patients Develop a Q9VF-specific CTL Response. It was important to determine whether a Q9VF CTL response is generated during HIV infection in humans. We performed a cross-sectional study on patients who had been infected for up to 16 yr. Virological and clinical characteristics of eight HIV-infected HLA-B7$^+$ patients are presented in Table I (individuals nos. 1–8). PBMCs were first analyzed in an IFN-γ ELISPOT assay. Incubation with peptides corresponding to well-characterized immunodominant HLA-B7–restricted HIV-1 Gag (Y10LF) and CMV (T10AM) epitopes induced significant IFN-γ release in seven individuals (Fig. 3 A). Interestingly, five out of the eight individuals (nos. 1–4 and no. 6) showed significant activation with Q9VF. As controls, PBMCs from seven HIV$^-$ HLA-B7$^+$ (nos. 9a–9g) individuals and one HIV$^+$ HLA-B7$^-$ (no. 10) individual were analyzed. None of them responded to Q9VF nor to the classical Gag Y10LF epitope (Fig. 3 A, individuals nos. 9a and 10). Differences between HIV$^+$ and HIV$^-$ HLA-B7$^+$ individuals were statistically significant (P < 0.05).

We documented the Q9VF response further in HLA-B7$^+$ persons by intracellular IFN-γ labeling and flow cytometry analysis (Fig. 3 B). IFN-γ production was detected in CD8$^+$ cells, and not in CD4$^+$ cells, from HIV-infected individuals, but not from an HIV$^-$ control, indicating that

Table I. *Patients' Characteristics*

Patient	Gender	Age	HLA–B7 status		Time since HIV detection	CD4 count[a]	Viral load[b]	Duration of ART	Treatment[c]
			FACS®	Sequence					
					yr	*cells/ml*		*yr*	
1	M	42	+	+	4	491	<50	4	d4T-ddI-NEV
2	M	33	+	+	9	642	1,776	5	3TC-d4T-NEV
3	M	54	+	+	2.5	446	<50	2.5	AZT/3TC
4	M	41	+	+	10	463	53,362	9	AZT/3TC-RIT-SAQ
5	M	49	+	+	16	616	18,815	15	d4T-ddI-NEV
6	F	47	+	ND	14	603	20,427	11	d4T-3TC
7	M	58	+	+	2.5	866	11,482	0	none
8	M	47	+	ND	10	289	22,383	6	3TC-d4T-IND
10	M	33	−	−	11	901	173	7	d4T-3TC

[a]Peripheral CD4$^+$ T lymphocyte count at the time of study.
[b]Copies of HIV-1 RNA per milliliter of plasma at the time of study.
[c]Treatment at the time of the study.
d4T, stavudine; ddI, didanosine; NEV, nevirapine; 3TC, lamivudine; AZT, zidovudine; RIT, ritonavir; SAQ, saquinavir; IND, indinavir; ART, antiretroviral therapy.

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pol

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4139 -     GTAAAAACAATACATACAGACAATGGCAGCAATTTCACCAGTACTACGGTTAAGGCCGCCTGTTGGTGG
Pol        V  K  T  I  H  T  D  N  G  S  N  F  T  S  T  T  V  K  A  A  C  W  W
Pol-ARFP                           M  A  A  I  S  P  V  L  R  L  R  P  P  V  G  G

4208 -     GCGGGAATCAAGCAGGAATTTGGAATTCCCTACAATCCCCAAAGTCAAGGAGTAG
Pol        A  G  I  K  Q  E  F  G  I  P  Y  N  P  Q  S  Q  G  V
Pol-ARFP   R  E  S  S  R  N  L  E  F  P  T  I  P  K  V  K  E  *
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env

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5890 -     GCTACAGAAAAATTGTGGGTCACAGTCTATTATGGGGTACCTGTGTGGAAGGAAGCAACCACCACTCTA
Env        A  T  E  K  L  W  V  T  V  Y  Y  G  V  P  V  W  K  E  A  T  T  T  L
Env-ARFP                              M  G  Y  L  C  G  R  K  Q  P  P  L  Y

5959 -     TTTTGTGCATCAGATGCTAAAGCATATGATACAGAGGTACATAATGTTTGGGCCACACATGCCTGTGTA
Env        F  C  A  S  D  A  K  A  Y  D  T  E  V  H  N  V  W  A  T  H  A  C  V
Env-ARFP   F  V  H  Q  M  L  K  H  M  T  Q  R  Y  I  M  F  G  P  H  M  P  V  Y

6028 -     CCCACAGACCCCAACCCACAAGAAGTAG
Env        P  T  D  P  N  P  Q  E  V
Env-ARFP   P  Q  T  P  T  H  K  K  *
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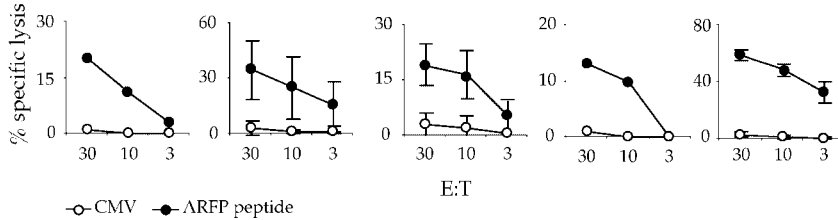
B

	Pol-ARFP		Env-ARFP		
	A9RL	S10PV	Q10QM	G10QT	M9PT
Relative Affinity	15	0.9	22	49	0.9
Responder	1/6	6/6	2/6	1/6	6/6

—○— CMV   —●— ARFP peptide

Figure 4. Identification of five potential epitopes derived from Pol⁻ and Env⁻ ARFPs. (A) Nucleotide and amino acid sequences of ARFPs encoded within the *pol* and *env* genes. Start and stop codons are in bold, and the Kozak sequence is underlined. Nucleotide numbering is according to HIV_{LAI} sequence. The predicted amino acid sequences of the corresponding polypeptides (Pol-ARFP and Env-ARFP) are shown, and HLA-B7 potential epitopes (A9RL, S10PV, Q10QM, G10QT, and M9PT) are underlined. (B) Peptide relative affinity for HLA-B7 and immunogenicity in HLA-B7 mice. Peptide relative affinity was calculated as described in Materials and Methods. A low relative affinity (<5) denotes a strong binding. To measure immunogenicity, six HLA-B7 mice were immunized with each peptide. Splenocytes were tested in a ^{51}Cr release cytotoxic assay using autologous target cells pulsed with the corresponding peptide or with a nonrelevant CMV peptide as a control. The number of responding mice is indicated. Data are mean ± SD of responder mice and are representative of two independent experiments. Cytolytic responses were considered positive when specific lysis was $>10\%$ at an effector/target (E/T) ratio of 30:1.

Q9VF is presented by MHC-I (Fig. 3 B). We also performed a ^{51}Cr release assay after two stimulations of PBMCs with either the Q9VF, T10AM, or G9AT peptides (Fig. 3 C). HIV-infected patients 1 and 2 displayed a strong lytic activity against T2-B7 target cells loaded with either Q9VF or the CMV-derived peptide, but did not respond to the HCV peptide. The HIV⁻ control donor only responded to the CMV peptide. Therefore, the Q9VF epitope derived from Gag-ARFP is generated during the natural course of HIV-1 infection, and approximately half of HIV-infected HLA-B7⁺ persons tested displayed a CTL response against it.

Identification of Additional ARFP-derived Epitopes. To extend these observations to other ARFPs, we selected two overlapping epitopes from Pol-ARFP, a polypeptide potentially encoded by a *pol* ORF +1 sequence (A9RL-AAISPVLRL and S10PV-SPVLRLRPPV-), and three epitopes from Env-ARFP, a polypeptide potentially encoded by *env* ORF +1 sequence (Fig. 4 A, Q10QM-QP-PLYFVHQM-, G10QT-GPHMPVYPQT-, and M9PT-MPVYPQTPT-). As for Q9VF, we tested these candidate peptides for HLA-B7 affinity and for immunogenicity.

The five peptides generated CTL responses in immunized HLA-B7$^{m\alpha3}$ mice, with efficiencies ranging from 1/6 to 6/6 responding mice (Fig. 4 B). We also asked whether these peptides were generated in HIV-infected individuals. As a positive control, a panel of peptides corresponding to known HIV epitopes, derived from Gag, Env, Nef, and Vpr, was similarly tested. The ELISPOT profile of IFN-γ responses varied between the eight HLA-B7⁺ HIV-infected patients; however, a response for at least one of the ARFP or traditional peptides was detected in each individual (Fig. 5 A). For each ARFP-derived epitope, a significant IFN-γ response was detected in PBMCs from at least five out of eight HIV-infected patients. PBMCs from control individuals (seven HLA-B7⁺ HIV⁻ and one HLA-B7⁻ HIV⁺) did not produce significant IFN-γ levels upon exposure to these peptides (Fig. 5 A). Comparing HIV⁺ and HIV⁻ HLA-B7⁺ individuals indicated that responses against each known or ARFP-derived epitope were statistically significant ($P < 0.05$). We further characterized the CTL responses from individuals 1 and 2. Cultured PBLs were stimulated twice with peptide and assayed for ^{51}Cr release. We observed a strong lytic
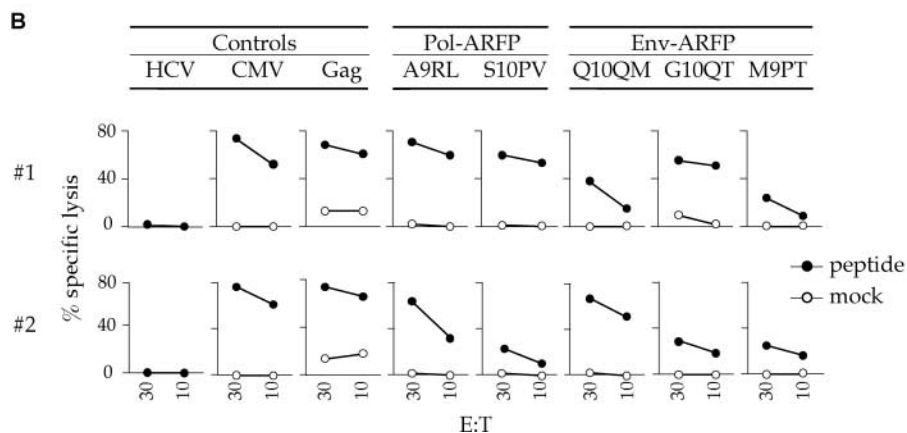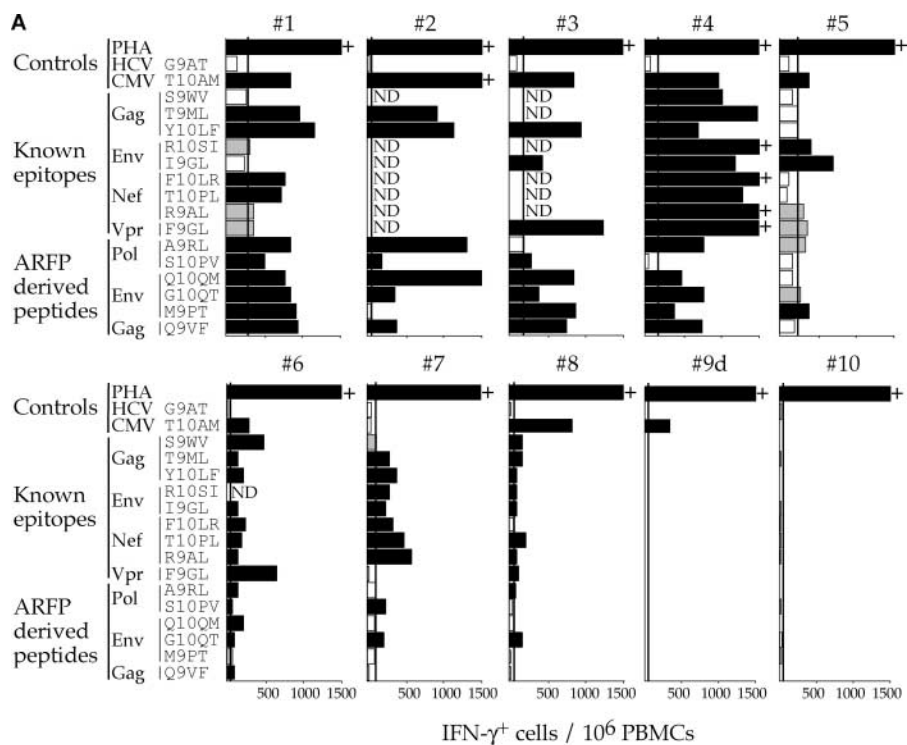
Figure 5. CTL responses to the six ARFP-derived epitopes in HIV-infected individuals. (A) IFN-γ ELISPOT assay was performed with the indicated peptides. PBMCs from eight HIV-infected HLA-B7+ patients (nos. 1–8) and, as controls, from seven HIV− HLA-B7+ (nos. 9a–9g) and one HIV+ HLA-B7− (no. 10) individuals were analyzed. For controls, responses against subjects 9d and 10 are shown (similar results were obtained for individuals 9a–9g for all HIV peptides tested; not depicted). Results are presented as IFN-γ+ cells/10^6 PBMCs. Responses were considered positive when IFN-γ production was >50 spots/10^6 PBMCs and exceeded at least twofold background levels (measured with the HCV peptide). (white bars) Negative responses (below background thresholds, which are represented by the black line). (gray bars) Weak responses (twofold background levels). (black bars) Strong responses (at least threefold background levels). (+) Responses ≥1,500 IFN-γ+ cells. ND, not determined. (B) Cytotoxic activity of PBMCs from two HIV-infected HLA-B7+ patients (nos. 1 and 2). ^{51}Cr release assay was performed after two rounds of stimulation of PBMCs with the indicated peptides. Specific lysis was measured by using T2-B7 cells pulsed with the corresponding peptides (closed circles) or with a HCV peptide (open circles, G9AT) as a control. Data are means of duplicates at the indicated effector/target (E/T) ratio, and are representative of two independent experiments. Cytolytic responses were considered positive when specific lysis was >10% at an effector/target (E/T) ratio of 30:1.

activity against target cells loaded with the different peptides (Fig. 5 B). Altogether, these results demonstrate the presence of an immune response against epitopes derived from Env-ARFP, Pol-ARFP, and Gag-ARFP in HIV-1–infected individuals.

Frequency of ARFP-derived Peptides in Naturally Occurring HIV Isolates. Next, we examined whether sequences corresponding to the six ARFP-derived peptides were conserved among the HIV-1 strains included in the Los Alamos database (Fig. 6 A). Because the peptides studied here were derived from the clade B HIV$_{LAI}$ strain, this analysis was first performed on clade B strains and then on all clade (A–C) isolates (Fig. 6 A). Except for Gag-ARFP, for which a stop codon within the ARF was frequently observed (22 and 46% for clade B and all clades, respectively), the reading frames encoding Pol-ARFP and Env-ARFP were generally open (96–99% in all clades). Thus, the majority of circulating HIV-1

strains could express at least one of these ARFPs. Moreover, the peptides that we selected in Pol- and Env-ARFP were relatively well conserved among viral isolates, especially in clade B (24–80%, depending on peptide; Fig. 6 A).

Notably, the Q9VF epitope (QPRS**D**THVF) from HIV$_{LAI}$ Gag-ARFP is not well conserved among other viral isolates because the Q9VF/5N (QPRS**N**THVF) encoding sequence is present in 68% of clade B strains (Fig. 6 A). Therefore, we asked whether Q9VF and Q9VF/5N-specific CTLs would cross-react with the alternate peptide. To this end, we derived mouse CTL lines against each peptide. The two peptides showed affinity for HLA-B0702 and were comparably immunogenic (unpublished data). In ^{51}Cr release assays, CTL lines specific for Q9VF and Q9VF/5N showed significant cross-reactivity with the alternate peptide, but not with a control CMV peptide (Fig. 6 B). Similar results were obtained by ELISPOT analysis (unpublished data).
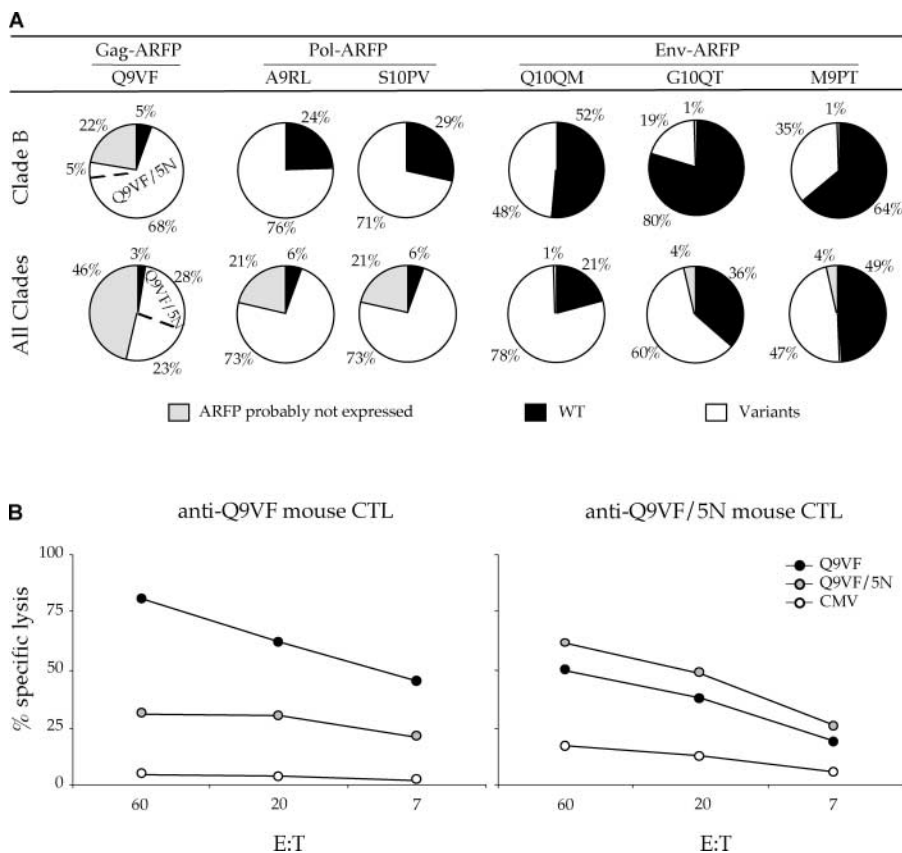
Figure 6. Conservation of ARFP-derived epitope sequences among circulating HIV-1 strains. (A) For each ARFP-derived peptide, the percentage of HIV isolates carrying the corresponding sequence is shown. Analysis was performed among clade B and all clade HIV-1 strains present in the Los Alamos database. Strains without ARF translation potential (absence of AUG start codon or presence of stop codons within the ARFP) are also indicated. The Gag-ARFP Q9VF (QPRS**D**THVF) has a major variant, Q9VF/5N (QPRS**N**THVF). WT, wild type, corresponding to the HIV$_{LAI}$ sequence. (B) Cross-reactivity between Q9VF and Q9VF/5N peptides. Six HLA-B7 mice were immunized with Q9VF or Q9VF/5N. Splenocytes were tested using autologous target cells pulsed with either Q9VF or Q9VF/5N peptide or with a nonrelevant CMV peptide as a control (R10TV). Data are mean of six mice and are representative of three independent experiments. Cytolytic responses were considered positive when specific lysis was >10% at an effector/target (E/T) ratio of 20:1.

Discussion

We report that various nontraditional viral epitopes are presented by MHC-I molecules in HIV-1–infected cells. We first identified in the HIV-1 genome 3 ARFs potentially encoding polypeptides from 34 to 52 amino acids in length (which we named Gag-ARFP, Pol-ARFP, and Env-ARFP). Various transcriptional and translational mechanisms, such as ribosomal frameshifting or internal AUG translation initiation, may account for the biosynthesis of these nontraditional polypeptides (for review see reference 25). For example, Gag-ARFP could be produced through a ribosomal frameshifting from the *gag* sequence. Although this remains to be formally proven, we noticed the presence of a conserved slippery site (UUUAAAU) upstream of the Gag-ARFP start codon that may promote the frameshifting (39). Of note, we were unable to detect the presence of Gag-ARFP in HIV-infected cells (unpublished data). This could be due to the poor sensitivity of the anti–Gag-ARFP polyclonal antibodies that we have generated (unpublished data). It is also conceivable that Gag-ARFP, as well as other ARFPs, are short-lived and/or expressed at low levels. These polypeptides could be related to defective ribosomal products that are prematurely terminated or misfolded polypeptides rapidly degraded by the proteasome (40, 41). Abrogating Gag-ARFP expression by inserting a premature stop codon within the ARF did not affect viral growth, indicating that this small protein is not essential for the viral life cycle. Determining how Gag-ARFP, Pol-

ARFP, or Env-ARFP are produced and understanding their biological function will require further investigations.

Using an algorithm, we defined six ARFP-derived epitopes putatively presented by the HLA-B7 molecule. Several lines of evidence indicate that these unconventional epitopes are of immunologic relevance. First, the six peptides that we selected are immunogenic in HLA-B7 mice. The repertoires of epitopes recognized by HLA-B7[mα3] mouse and by human T cells are comparable in their breadth (26); thus, this murine model offers a rapid and convenient system to assess immunogenicity of various antigens, including peptides. Second and most importantly, the six epitopes are generated and recognized in HIV-1–infected patients. This was demonstrated by ex vivo IFN-γ ELISPOT and intracellular labeling of patient PBMCs, as well as by a ^{51}Cr release test performed after a short period of PBMC stimulation, which confirmed the killing activity of peptide-stimulated cells. With each of the six ARFP-derived peptides, a significant response was observed in ∼50% of the HLA-B7[+] HIV-infected patients analyzed. The breadth of the response varied between each individual, ranging from 1/6 to 6/6 peptides recognized. Third, we show that the Gag-ARFP–derived epitope Q9VF is generated in vitro in HIV-infected cells because infected cells were recognized by Q9VF-specific CTLs. Moreover, the Gag-ARFP negative mutant HIV strain carrying a premature stop codon was no longer able to activate these

CTLs, indicating that Q9VF is generated from the expected ARF. It will be worth determining whether the other ARFP-derived epitopes are similarly presented by HIV-infected cells in culture. Altogether, these results indicate that various nontraditional epitopes are presented by MHC-I molecules in HIV-1–infected cells, leading to the activation of specific CTLs.

A comprehensive analysis of naturally occurring viral sequences in the Los Alamos database revealed various patterns of intraclade and interclade conservation of the six selected peptides. Some peptides are highly conserved (64 and 80% intraclade B conservation for Env-ARFP–derived M9PT and G10QT epitopes, respectively). Others are less frequently encountered, such as Pol-ARFP–derived A9RL and S10PV epitopes (24 and 29% intraclade B conservation, respectively). The Gag-ARFP–derived Q9VF peptide is infrequent among viral isolates, but its cross–reactive variant Q9VF/5N is found in up to 68% of clade B viruses. We have also performed a preliminary analysis of the provirus sequences encountered in three of the HLA-B7$^+$ patients studied here. In agreement with the analysis of the Los Alamos database, we observed in these patients a high variability of Gag-ARFP sequences, which encoded mostly Q9VF/5N, and more rarely Q9VF or other variant epitopes (unpublished data). Altogether, these results indicate that ARFPs may generate either conserved or variable epitopes, similar to primary ORF-encoded epitopes. It is important to note that the observed conservation of a given ARF sequence will depend not only on immunological and virological selection pressures exerted on its ARFP, but also on the protein encoded by the corresponding primary ORF.

We have limited our analysis to epitopes presented by HLA-B7 haplotypes and encoded by sequences beginning with AUG and preceded by a consensus Kozak sequence. One can expect that other nontraditional epitopes may be presented by various HLA molecules. Different cryptic translation mechanisms, such as non-AUG translation initiation (42), have been reported in other systems and may be operative in HIV-1–infected cells as well (for review see reference 43). Therefore, the repertoire of viral epitopes is certainly much broader than previously thought (44). Although these nontypical epitopes may be infrequent, we provide evidence here that they are generated during natural HIV-1 infection. This underappreciated source of epitopes should be studied more extensively (e.g., by longitudinally monitoring CTL responses during the course of HIV infection. Nontypical epitopes should be taken into account when designing candidate vaccine formulations.

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