Human immunodeficiency virus type 1 has an additional coding sequence in the central region of the genome

(acquired immune deficiency syndrome/bacterial expression/open reading frame U)

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ABSTRACT Eight coding regions designated *gag*, *pol*, *env*, *sor*, *R*, *tat*, *art/trs*, and *3'orf* have been identified in the genome of the human immunodeficiency virus type 1 (HIV-1). Several other open reading frames have the potential to encode additional viral proteins. In this study, we show that HIV-1 has another coding sequence whose product is expressed during natural infection. Unlike antibody to other HIV-1 proteins, the prevalence of antibody to the product encoded by this region is elevated in patients with acquired immune deficiency syndrome (AIDS). Because no analogous coding region has been identified in HIV-2, the antibody to the product of this coding region may serve as a marker to distinguish infection with HIV-1 from infection with HIV-2.

Human immunodeficiency virus type 1 (HIV-1), formerly called human T-lymphotropic virus type III (HTLV-III)/lymphadenopathy-associated virus (LAV), or AIDS-related virus (ARV), is the causative agent of acquired immune deficiency syndrome (AIDS) and related disorders (1–4). HIV-1 is an exogenous nononcogenic retrovirus with a complex genomic organization. In addition to the three genes (*gag*, *pol*, and *env*) commonly found in replication-competent animal retroviruses, five additional genes have been identified. Three of them, *3'orf*, *sor*, and *R* were initially predicted from DNA sequence analysis (5–8) and have been shown to encode products that induce antibody responses during natural infection (9–12). The *3'orf* gene-encoded protein p27 has characteristics of a GTP binding protein and was reported to be a negative regulator of virus replication (13–15). The *sor* gene encodes a 23-kDa protein and HIV-1 *sor* mutants were known to have impaired infectivity (16, 17). The presence of an *R* gene was suggested by the observation that sera from HIV-1 infected individuals contained antibody reactivity to a recombinant R protein (12). The natural product and the function of *R* remain unknown. The *tat* and *art/trs* genes were identified from functional studies (18–21). They are composed of three exons; their second coding exons overlap the *env* gene but use different reading frames. Both *tat* and *art/trs* genes are requisite for viral replication (20–23). The *tat* product is a 14-kDa protein that regulates expression of viral genes at the transcriptional and/or posttranscriptional level (24–29). The *art/trs* gene encodes a product of ≈19 kDa that is required for the expression of virus structural proteins (20, 21, 30).

Despite the unusually large number of genes involved in the life cycle of HIV-1, there are many other open reading frames in the genome that may be considered candidate regions to encode additional virus proteins. For instance, a small open reading frame (*orf*) flanked by the *tat* and *art/trs*

first coding exons and the *env* gene is conserved among several HIV-1 isolates (31). This *orf*, termed U in one previous report, has the potential to encode a protein of ≈80 amino acids (32). In this study, we present evidence to show that *orf* U is another coding sequence of HIV-1, and its product induces an antibody response during natural infection.

MATERIALS AND METHODS

Plasmids. All DNA manipulation was according to the standard techniques (33). Plasmid pRTUA (Fig. 1) was constructed by cloning a 2190-base-pair *Kpn* I/*Kpn* I fragment from the HTLV-III clone BH10 into pUC18 (6). A 194-base-pair *Ssp* I/*Sma* I fragment from pRTUA was ligated with *Nco* I linkers d(CCCATGGG) (New England Biolabs) and then cloned into the *Nco* I site of a vector pXVR (34). After transfecting *Escherichia coli* X-90 [*ara* Δ(*lac-pro*) *nalA* *argE*(Am) *thi* *Rif*^r *F'*(*lac*⁺-*pro*⁺ *lacI*^{q1})] by the calcium chloride method, clones with insert DNA in the right orientation were selected by restriction mapping (36). One of these clones, pUSS19, was used in this study. For DNA sequencing, a *Hind*III/*Kpn* I fragment of pUSS19, which contains the upstream junction region between v-Ha-ras and the HIV-1 DNA insert, was cloned into phage M13 and sequenced by the chain termination method (35).

Sera. Serum specimens used in this study included 137 sera from HIV-1-infected individuals whose clinical status had been established by the Walter Reed (WR) clinical staging system (37). Antibody reactivity to the recombinant peptide expressed by pUSS19 was determined in 136 sera because one sample did not have sufficient quantity. Twenty-seven HIV-2-positive sera from West Africans, and 20 HIV-1-negative sera from healthy blood donors were also included in this study. A monoclonal antibody to v-Ha-ras p21 was a gift of B. T. Pan (Harvard Medical School). Some sequential serum samples from HIV-1 seropositive people were kindly provided by the National Institute of Allergy and Infectious Diseases funded Multi-Center AIDS Cohort Study (38).

Antigen Preparation and Immunodetection. An overnight culture of *E. coli* X-90 carrying plasmid pUSS19 was inoculated into Luria-Bertani medium supplemented with ampicillin (50 μg/ml) at a 1:20 dilution. Induction with 5 mM isopropyl β-D-thiogalactopyranoside (IPTG) was started when OD₅₅₀ reached 0.25. After growing the cells at 37°C with vigorous aeration for 3 hr, cells were pelleted (4200 rpm, 20 min at 4°C, Beckman Accuspin FR) and washed once with buffer containing 10 mM Tris-HCl (pH 7.5) and 50 mM NaCl. After washing, the cell pellet was resuspended in buffer

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Abbreviations: HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; AIDS, acquired immune deficiency syndrome; IPTG, isopropyl β-D-thiogalactopyranoside.

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FIG. 1. (A) The location of the orf U and its relationship to the other genes of HIV-1 in plasmid pRTUA. Plasmid pRTUA contains a 2190-base-pair *Kpn I/Kpn I* fragment from HTLV-III DNA clone

containing 0.1 M dithiothreitol, 0.08 M Tris-HCl (pH 6.8), 2% NaDodSO₄, 10% (vol/vol) glycerol, and 0.001% bromophenol blue (whole cell lysate) and separated on 13% NaDodSO₄/polyacrylamide gel. NaDodSO₄/PAGE was done according to the Laemmli method (39). The procedure for partial purification of the protein expressed by pUSS19 was modified from that of Pallas *et al.* (36). Pelleted cells were resuspended in 20 times the cell volume of bacterial lysis buffer (25 mM Tris-HCl, pH 8.0/1 mM phenylmethylsulfonyl fluoride) and sonicated six times, 45 sec each, on ice. The sonicated suspension was centrifuged for 5 min at 13,000 × *g* at 4°C. The pellet was washed sequentially with bacterial lysis buffer, bacterial lysis buffer containing 1% Nonidet P-40 and 2 mM 2-mercaptoethanol, and bacterial lysis buffer containing 2 M NaCl and 2 mM 2-mercaptoethanol. After each wash, the pellet suspensions were sonicated for 1 min and centrifuged at 13,000 × *g* at 4°C for 5 min. The pellet was then resuspended in 25 mM Tris-HCl (pH 8.0) containing 3 M urea and incubated on ice for 1 hr. After sonication for 1 min, the insoluble fraction (partially purified recombinant peptide) was separated on NaDodSO₄/polyacrylamide gel. After electrophoresis, proteins were transferred to the nitrocellulose membrane (Schleicher & Schuell) by passive transfer. The amount of recombinant U peptide on each strip is ≈0.25 μg. The procedure for immunoblotting analysis has been described (40).

HIV-1 antigens used for immunoblotting analysis in this study were prepared from a culture supernatant of a HTLV-III_B-infected Molt-3 cell line (2, 10). The presence of antibody against HIV-1 viral antigens was also determined by radioimmunoprecipitation and NaDodSO₄/PAGE as described (10). For this assay, the cell lysates prepared from HIV-1-infected Molt-3 cells labeled with L-[³⁵S]cysteine (>22.2 TBq/mmol) or L-[³⁵S]methionine (>29.6 TBq/mmol) (New England Nuclear) were used as antigens.

RESULTS AND DISCUSSION

Fig. 1 shows the HIV-1 DNA sequence in the orf U, which was cloned into the vector pXVR, and the steps involved in the construction of plasmid pUSS19. The reading frame in the junction region between preceding v-Ha-ras and the HIV-1 DNA insert was further verified by DNA sequencing (Fig. 1). The recombinant protein expressed by pUSS19 is expected to contain the first 111 amino acids of v-Ha-ras, the last 49 amino acids of orf U, and 1 extra glycine residue at the junction between the preceding v-Ha-ras and the HIV-1 insert DNA.

Upon induction by IPTG, a recombinant protein of ≈20 kDa was detected in the lysate of *E. coli* carrying pUSS19 (Fig. 2). Because of its relative insolubility, partial purification of the protein designated recombinant U peptide in this report can be readily achieved (Fig. 2). The recombinant U peptide was specifically recognized by a monoclonal antibody to v-Ha-ras p21 and was not detected in lysate of uninduced cells by immunoblotting analysis (data not shown). The expression level was markedly diminished when the

BH10 (6). The orf U sequence of BH10 is shown. The nucleotide number is according to that of Ratner *et al.* (6). kb, Kilobases. (B) Steps involved in the construction of pUSS19. A 194-base-pair (bp) *Ssp I/Sma I* restriction fragment from plasmid pRTUA was ligated with *Nco I* linkers and cloned into the *Nco I* site in pXVR (34). Amp^r, ampicillin-resistance gene; tac, tac promoter; Ori, origin of replication; B, *Bam*HI; H, *Hind*III; K, *Kpn* I; N, *Nco* I; P, *Pst* I; Sa, *Sal* I; Sm, *Sma* I; Ss, *Ssp* I; X, *Xba* I; TAG, stop codon. (C) The sequence of the junction between the preceding v-Ha-ras and orf U was determined by the chain termination method (35). The deduced amino acid sequence is also shown. One extra glycine residue was introduced at the beginning of the orf U sequence in this construct.

FIG. 2. (A) Expression of recombinant U peptide. NaDodSO₄/PAGE analysis of whole cell lysates from IPTG-induced X-90 culture (lane 3, indicated by I) and its uninduced control (lane 2, indicated by U). Lane 4 shows partially purified recombinant U peptide. Recombinant U peptide indicated by arrow. Molecular markers are shown in kDa (lane 1). (B and C) Antibody reactivity to the recombinant U peptide. Immunoblot analyses were carried out with whole cell lysate from uninduced controls and IPTG-induced cultures (B), and partially purified recombinant U peptide (C). Lanes 1-3, representative sera from seronegative blood donors; lanes 4-9, representative sera from HIV-1 seropositive donors. In B, cell lysate from IPTG-induced culture is indicated by +, and - indicates uninduced controls. In C, all cell lysates were from induced cultures. Position of the recombinant peptide U is indicated by arrow.

recombinant U peptide was expressed without fusion (data not shown).

The question of whether the HIV-1 DNA insert in pUSS19 contains a coding sequence of HIV-1 was addressed by screening HIV-1 antibody-positive human sera for antibody reactivities to the recombinant U peptide. Representative immunoblotting profiles are shown in Fig. 2. Among the 136 HIV-1 antibody-positive human sera tested, 43 had detectable antibody to the recombinant U peptide (Fig. 2 B and C, lanes 7-9). No reactivity to the recombinant U peptide was detected with sera from 20 HIV-1 antibody-negative blood donors (Fig. 2 B and C, lanes 1-3) or with 27 HIV-2 antibody-positive human sera (data not shown). Reactivity to the recombinant U peptide was unlikely to be directed to the v-Ha-ras portion of the fusion peptide since no reactivity with v-Ha-ras p21 expressed by plasmid pXVR was detected in 20 randomly selected human sera that tested positive for antibodies to the recombinant U peptide (data not shown). These results indicate that at least some part of the HIV-1 sequence in the pUSS19 is expressed during natural infection.

A cross-sectional study was conducted to determine whether antibody to the recombinant U peptide is more likely to be detected at some particular clinical stages of HIV-1 infection. For this analysis, sera from 136 HIV-1-positive individuals whose clinical status had been previously staged by the WR clinical staging classification system were used (37). As shown in Fig. 3, the distribution of antibody to recombinant U peptide appears to be bimodal with one peak associated with early stages (WR2 and WR3) of infection and the other associated with the late stage (WR6) of infection. The elevation of antibody prevalence in stage 6 is unique when compared with the prevalence of antibodies to other viral antigens, such as *gag* gene-encoded p24, *sor* gene-encoded p23, and *3'orf*-encoded p27, all standardized with the same panel of sera (Fig. 3).

Our data provide evidence for the presence of another coding sequence located in the central region of the HIV-1 genome. The exact initiation codon of this HIV-1 gene remains to be determined. One possibility is that the first methionine codon found after a known splice acceptor site [nucleotide 5557, according to Muesing *et al.* (8)] of HIV-1 is

the initiator. This putative initiator codon is conserved in the orf U in several HIV-1 isolates (31). We have detected a HIV-1-specific protein of ≈ 16 kDa in HuT 78 cells transfected with a HIV-1 infectious clone, HX10, which has an initiation codon ATG in the orf U (41), by using a goat antiserum raised against the recombinant U peptide (unpublished observation). The molecular weight of this protein is larger than the size predicted from the sequence of the orf U.

FIG. 3. Prevalence of antibodies to recombinant U peptide, p27, p23, and p24 in different clinical stages of HIV-1 infection. Vertical axis shows the prevalence rates of antibodies to recombinant U peptide, p27, p23, and p24. Horizontal axis shows five WR clinical stages. Antibody to recombinant U peptide was analyzed by the immunoblot technique and antibodies to other HIV-1 antigens were scored by radioimmunoprecipitation and NaDodSO₄/PAGE assay. The number (n) of patients in each clinical stage is indicated below each column. One serum from a patient classified at WR3 did not have sufficient quantity for assaying antibody reactivity to recombinant U peptide.

FIG. 4. Antibody reactivity to the recombinant U peptide detected in sequential serum samples from a HIV-1-seropositive patient. The antigen used in A was HIV-1 viral lysate prepared from the supernatant of HTLV-III_B-infected Molt-3 cell line. The antigen used in B was partially purified recombinant U peptide. Antibody reactivities of four sequential serum samples taken at 6-month intervals are shown in sequence from lane 1 to lane 4. The patient was diagnosed with AIDS at the visit when the serum specimen shown in lane 4 was collected. Procedures for immunoblot analysis were as described.

This raises the possibility that either a more complex splicing pattern is involved in the expression of orf U or the native U protein undergoes extensive posttranslational modification. However, if the latter explanation is correct, it suggests that the product of this gene is dispensable for viral replication, because an infectious clone HXB2 (41) lacks the putative initiation codon of orf U.

In the natural course of infection, HIV-1 is more readily isolated from patients with AIDS (WR6) (R.R., unpublished observation). WR6 patients in this study showed a higher prevalence of antibody to the recombinant U peptide. In agreement with the elevated rate of recombinant U peptide antibody in patients at the late stage of HIV-1 infection, one seropositive patient who was sequentially analyzed developed recombinant U peptide antibody 12–18 months prior to the development of AIDS. This antibody reactivity persisted throughout the follow-up (Fig. 4). These observations raise the possibility that antibody to the recombinant U peptide is developed during and/or after some active rounds of viral replication.

The lack of antibody reactivity to the recombinant U peptide in 27 HIV-2-positive sera tested is consistent with the observation that the HIV-1 coding sequence identified in this study has no counterpart in HIV-2 (42). This feature should allow the use of antibody reactivity to the U peptide to help confirm infections by HIV-1-related viruses among West Africans whose sera have antibody reactivity to both HIV-1 and HIV-2 (43).

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