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Identification of a Novel Retroviral Gene Unique to Human Immunodeficiency Virus Type 2 and Simian Immunodeficiency Virus SIV_{MAC}

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Human and simian immunodeficiency-associated retroviruses are extraordinarily complex, containing at least five genes, *tat*, *art*, *sor*, *R*, and 3′ *orf*, in addition to the structural genes *gag*, *pol*, and *env*. Recently, nucleotide sequence analysis of human immunodeficiency virus type 2 (HIV-2) and simian immunodeficiency virus SIV_{MAC} revealed the existence of still another open reading frame, termed X, which is highly conserved between these two viruses but absent from HIV-1. In this report, we demonstrate for the first time that the X open reading frame represents a functional retroviral gene in both HIV-2 and SIV_{MAC} and that it encodes a virion-associated protein of 14 and 12 kilodaltons, respectively. We also describe the production of recombinant TrpE/X fusion proteins in *Escherichia coli* and show that sera from some HIV-2-infected individuals specifically recognize these proteins.

Since the discovery of human immunodeficiency virus type 1 (HIV-1) as the etiological agent of acquired immunodeficiency syndrome in the United States, Europe, Central Africa, and other areas worldwide (4, 24, 25), additional retroviruses have been discovered to cause immunodeficiency in human and primate populations. These include human immunodeficiency virus type 2 (HIV-2), the second major class of human AIDS retroviruses endemic in West Africa (3, 7, 8), and the group of simian immunodeficiency viruses (SIV; for a review, see reference 28), which have been isolated from rhesus macaques (SIV_{MAC}; 9, 16, 18), African green monkeys (SIV_{AGM}; 23), and sooty mangabeys (SIV_{SMM}; 11, 21). HIV-1, HIV-2, and SIV all share similar ultrastructural, antigenic, and in vitro biological properties, including a tropism for CD4-bearing target cells and the ability to exhibit extensive in vitro cytopathicity in appropriate target cells (4, 7, 9, 11, 23, 24). Epidemiological studies, however, indicate that host range, disease association, and overall in vivo pathogenic potential can vary substantially among these viruses and raise the question of whether strain-specific genetic differences exist among the major viral groups which are of pathophysiological and clinical relevance (6, 15, 18a, 22, 24).

Nucleotide sequence comparisons of HIV-1, HIV-2, and SIV_{MAC} have demonstrated an overall highly conserved genomic organization, represented by long terminal repeat-*gag*-*pol*-*sor*-central region-*env*-3′ *orf*-long terminal repeat (1, 5, 12, 20, 26, 27, 30). HIV-2 and SIV_{MAC} are approximately 75% homologous at the nucleotide sequence level, and each is approximately 45% similar to HIV-1 (5, 12). Interestingly, there is an open reading frame in the genomes of HIV-2 and SIV_{MAC} that is missing in HIV-1. This open reading frame, termed X, is situated in the central viral region between the *sor* and *R* genes and partially overlaps the *sor* open reading frame on its 5′ end (5, 12). The deduced amino acid sequence of X predicts a protein of 112 amino acids and 14.5-kilodalton (kDa) calculated molecular mass which is conserved in 94 of 112 amino acid residues between HIV-2 and SIV_{MAC} (Fig. 1). In this study, we chemically synthesized HIV-2 and SIV_{MAC} X-specific oligopeptides, raised heterologous antisera to these peptides in rabbits, and expressed the SIV_{MAC} X open reading frame as TrpE fusion proteins in *Escherichia coli*. With these reagents, we have demonstrated that X encodes a novel retroviral protein uniquely present in HIV-2 and SIV_{MAC} and have provided evidence for its expression and immunogenicity in vivo.

Two synthetic peptides encompassing 19 residues from the N terminus of the deduced X amino acid sequences of HIV-2/ROD (7, 12) and SIV_{MAC}/PK82 (13, 14, 18) (underlined in Fig. 1) were synthesized and used to generate rabbit immune sera. The peptides were prepared by solid-phase methodology on a peptide synthesizer (model 430-A; Applied Biosystems) using a paramethylbenzhydrolamine resin (17). Synthetic peptides were coupled to keyhole limpet hemocyanin at the carboxy terminus after the addition of two glycine spacers and a cysteine residue. New Zealand White rabbits were immunized with 1 mg of keyhole limpet hemocyanin-coupled peptides emulsified 1:1 in Freund complete adjuvant, boosted twice at 2-week intervals with the same peptides mixed 1:1 with Freund incomplete adjuvant, and bled 6 weeks after the first immunization to collect immune sera. A peptide enzyme-linked immunosorbent assay with oligopeptides not coupled to keyhole limpet hemocyanin as the antigens was performed to confirm high-titer antipeptide antibodies in these sera (10).

The SIV_{MAC} X open reading frame was also expressed as a fusion protein in *E. coli*. An *Xho*II-*Hin*dIII fragment containing the entire SIV_{MAC} X open reading frame minus the first six nucleotides (SIV_{MAC} clone PKE102; 14) was subcloned into the bacterial expression vector pATH (29) immediately adjacent to and in frame with the bacterial *trpE* gene (pATH-X, Fig. 2a). *E. coli* HB101 transformed with pATH-X was treated in mid-log-phase growth with 3β-indole acrylic acid to induce the *trp* operon (22). Cell extracts were

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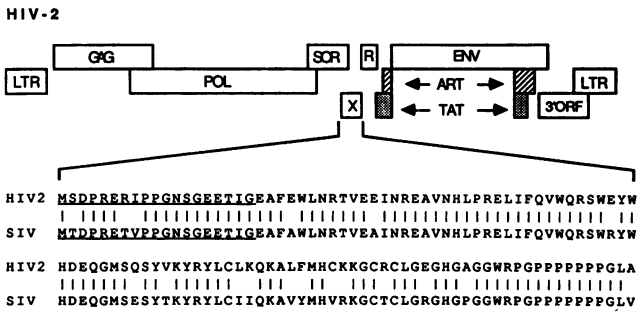

FIG. 1. Alignment of the deduced X amino acid sequence of HIV-2 and SIV_{MAC}. The genomic organization of HIV-2 is depicted as reported by Guyader et al. (12). Shown are eight open reading frames known to encode HIV proteins as well as the novel X open reading frame. A comparison of the deduced X amino acid sequence is shown below for HIV-2 (isolate ROD [12]) and SIV_{MAC} (isolate PK82 [14, 18]). Vertical bars indicate sequence identity. The first 19 residues (underlined in the HIV-2 and SIV_{MAC} X sequences) represent the oligopeptides which were synthesized to raise rabbit anti-X immune sera.

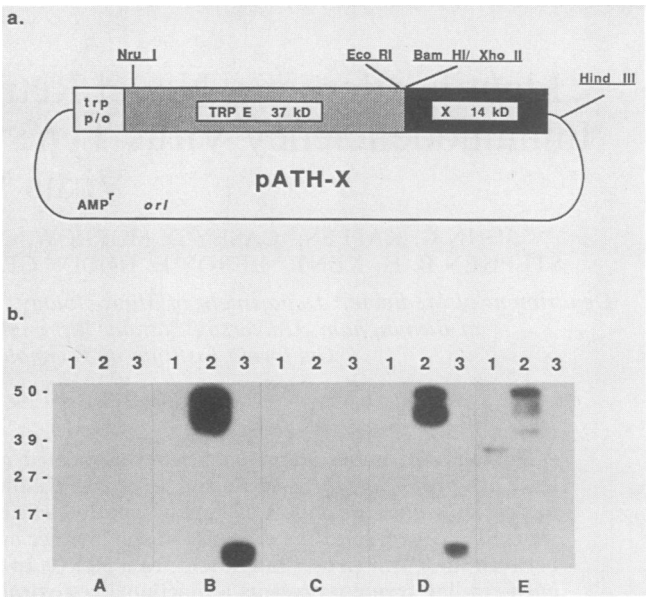

FIG. 2. Procaryotic expression of the SIV_{MAC} X protein. (a) Construction of pATH-X. A procaryotic expression vector, pATH, (29) was used to express the entire SIV_{MAC} X open reading frame minus the first two amino acid residues. A 422-base-pair *Xho*II-*Hin*dIII fragment of SIV_{MAC} clone PKE102 (14) was inserted into pATH at the *Bam*HI and *Hin*dIII sites as depicted. This placed the SIV_{MAC} X open reading frame in frame and downstream of the bacterial *trpE* gene to allow its expression as a 50-kDa fusion protein. A unique *Nru*I-*Eco*RI site was subsequently used to remove 95% of the *trpE* coding region, leaving only 16 TrpE-derived amino acids on the 5′ end of the expressed SIV_{MAC} X protein. This construct yielded a smaller fusion protein of 15 kDa. The position of the *trp* promoter-operon within the construct is indicated. (b) Western blot analysis of bacterially expressed SIV_{MAC} X proteins. *E. coli* cell lysates transformed with nonrecombinant vector pATH (lanes 1), pATH-X (lanes 2), and the ΔTrpE/X construct (lanes 3) were tested by immunoblot analysis for reactivity to rabbit sera immunized with SIV_{MAC} and HIV-2 X peptides (B and D lanes, respectively) as well as to preimmune rabbit sera (A and C lanes). A 50-kDa TrpE/X fusion protein and a 15-kDa ΔTrpE/X fusion protein were recognized only by X peptide immune sera. (A smaller band of approximately 40 kDa probably represents a prematurely truncated version of the 50-kDa trpE/X protein.) An antiserum raised against the bacterial *trpE* gene product (E lanes) was used as a control to confirm the size of the 50-kDa TrpE/X fusion protein (lane 2) and to identify the nonrecombinant 37-kDa TrpE protein (lane 1). This antiserum failed to detect the 15-kDa ΔTrpE/X protein (lane 3). Antigen-antibody complexes were detected by using ^{125}I-labeled protein A.

subjected to polyacrylamide gel electrophoresis (19), and a predominant protein of approximately 50 kDa, consistent with the predicted molecular mass of a TrpE/X fusion protein, was identified by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels (data not shown). A second plasmid construct was prepared by deleting an internal *Nru*I-*Eco*RI fragment in pATH-X, which removed 95% of the *trpE* coding sequence (Fig. 2a). This plasmid encoded only 16 amino acid residues derived from the *trpE* gene on the N terminus and resulted in an expressed X fusion protein of 15 kDa (ΔTrpE/X). Both the TrpE/X fusion protein and the ΔTrpE/X fusion protein constituted approximately 20% of the total cellular protein as judged by Coomassie blue staining of gel-electrophoresed bacterial lysates (data not shown). Sera from rabbits immunized with the SIV_{MAC} and HIV-2 X oligopeptides (Fig. 2b, B and D lanes, respectively) reacted strongly with these lysates on Western blots (immunoblots) (Fig. 2b, lanes 2 [TrpE/X] and 3 [ΔTrpE/X]), but no reactivity was detectable with the corresponding preimmune sera (Fig. 2b, A and C lanes) or with lysates of cultures transformed with the nonrecombinant vector (Fig. 2b, lanes 1). An anti-TrpE antiserum, used for control, detected both the 37-kDa TrpE protein alone as well as the 50-kDa TrpE/X fusion protein but did not recognize the 15-kDa ΔTrpE/X protein (Fig. 2b, E lanes).

To identify the putative X protein in SIV_{MAC}- and HIV-2-infected cells and cell-free virions, immunoblotting techniques were used to probe viral preparations for reactivity with the rabbit immune sera. Figure 3a shows the Western blot patterns of viral preparations from cultures infected with HIV-1 (lanes 1), HIV-2 (lanes 2), and SIV_{MAC} (lanes 3) and from mock-infected cells (lanes 4). A polyclonal human anti-HIV-1 serum (RR lane) and a polyclonal human anti-HIV-2 serum (ST lanes) were used as controls to confirm the presence of virus-specific antigens. Probing with the X-specific rabbit immune sera (anti-SIV_{MAC}, Fig. 3, B lanes; anti-HIV-2, Fig. 3, D lanes) resulted in the identification of 14- and 12-kDa viral proteins in the HIV-2 and SIV_{MAC} viral preparations, respectively, but not in similar preparations of HIV-1 or virally uninfected control cultures. Similar results were obtained when whole-cell lysates infected with these viruses were used. No reactivity was detected with the preimmune sera (A and C lanes). Interestingly, sera from rabbits immunized with the HIV-2 X peptide recognized only the homologous HIV-2 X protein (D lanes), while the SIV_{MAC} X peptide antisera recognized both the HIV-2 and the SIV_{MAC} X proteins (B lanes). The SIV_{MAC} X protein appeared to be slightly smaller than the HIV-2 X protein and was reproducibly present in considerably smaller amounts. The significance of these differences is presently under study.

To determine whether the X protein was actually virion associated, HIV-2 virus was first concentrated by ultracentrifugation and then was further purified on a 20 to 60% continuous sucrose gradient prior to Western blot analysis (Fig. 3b). HIV-2 X peptide immune sera detected the identical 14-kDa X protein on immunoblots of banded HIV-2 virions (Fig. 3b, D lane). The intensity of the X-specific band

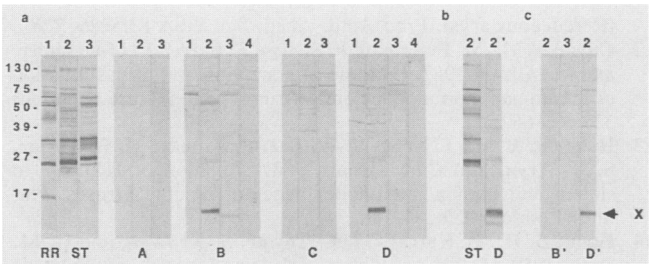

FIG. 3. Identification of the HIV-2 and SIV_{MAC} X proteins. (a) Immunoblot of viral preparations probed with X peptide immune and preimmune sera. HIV-1 (isolate IIIb [24]; lanes 1), HIV-2 (isolate ST [18a]; lanes 2), SIV_{MAC} viral lysates (isolate PK82 [14, 18]; lanes 3), and lysates of uninfected cell culture supernatants (lanes 4) were separated on a 12.5% polyacrylamide–sodium dodecyl sulfate gel, electrophoretically transferred to nitrocellulose, and reacted with the following sera diluted 1:100 in phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20: human anti-HIV-1-positive serum (RR), human anti-HIV-2-positive serum (ST), rabbit SIV X peptide preimmune (A) and immune (B) sera, and rabbit HIV-2 X peptide preimmune (C) and immune (D) sera. Bound antibody was detected by reaction with horseradish peroxidase-conjugated anti-rabbit and anti-human immunoglobulins with diaminobenzidine as a substrate. (b) Immunoblots of sucrose-banded, purified HIV-2 virions. HIV-2 viral supernatants (isolate ST [18a]) were clarified by low-speed centrifugation, and virus was pelleted through a 20% sucrose cushion and then banded in a 20 to 60% continuous sucrose gradient. Immunoblots were prepared as described previously (3), and identical strips containing purified HIV-2 virions as antigen (2') were reacted with an anti-HIV-2-positive human serum (ST) and the rabbit HIV-2 X peptide immune sera (D). (c) Competitive adsorption assay using bacterially expressed SIV_{MAC} X protein to adsorb anti-X antibodies. Bacterial lysates of pATH-X-transformed *E. coli* immobilized on nitrocellulose were incubated with SIV_{MAC} and HIV-2 X peptide immune sera (B' and D', respectively). Preadsorbed sera were subsequently tested by Western blot analysis for reactivity with preparations of HIV-2 (isolate ST [18a]; lanes 2) and SIV_{MAC} (isolate PK82 [14, 18]; lane 3).

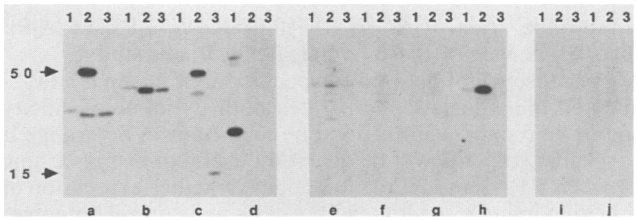

FIG. 4. Analysis of human sera for reactivity with the SIV_{MAC} X proteins expressed in *E. coli*. Cell lysates transformed with the pATH vector (lanes 1), pATH-X (lanes 2), and the ΔTrpE/X construct (lanes 3) were separated on a 12.5% polyacrylamide–sodium dodecyl sulfate gel, transferred to nitrocellulose, and analyzed by immunoblotting for reactivity to four HIV-2 antibody-positive human sera (a to d), four HIV-1-positive human sera (e to h), and two normal control sera (i and j). Sera were diluted 1:50 in phosphate-buffered saline containing 5% nonfat dry milk and 0.1% Tween 20. Arrows denote the positions of the 50-kDa TrpE/X and 15-kDa ΔTrpE/X fusion proteins recognized by two of four HIV-2-positive human sera (a and c). Bound antibody was detected by ^{125}I-labeled protein A.

was equal to or slightly greater than those from cell lysates infected with comparable amounts of HIV-2 (data not shown). A human serum (Fig. 3b, ST lane) from a healthy HIV-2-infected individual and the preimmune rabbit antiserum failed to detect the X protein in the same antigen preparation. These data were confirmed with a second HIV-2 isolate (HIV-2/ROD; 7).

Identification of the 12- and 14-kDa proteins as the virally encoded X gene products was further established by competitive adsorption assays (Fig. 3c). SIV_{MAC} and HIV-2 X immune sera were adsorbed with nitrocellulose-immobilized lysates of *E. coli* transformed either with the pATH cloning vector or with the pATH-X recombinant plasmid. Adsorption of the SIV_{MAC} X immune sera with the 50-kDa TrpE/X fusion protein resulted in a complete loss of antibody reactivity with both HIV-2 (Fig. 3c, B' lanes) and SIV_{MAC} viral preparations. Preadsorption of the same sera with *E. coli* lysates transformed with the pATH vector alone gave hybridization signals equal to that in Fig. 3a, B lanes (not shown). The reactivity of HIV-2 X peptide antisera was only slightly diminished by preadsorption with the SIV_{MAC} TrpE/X fusion protein (Fig. 3c, D' lane), as was expected, since this antiserum had failed to recognize the heterologous X protein on previous immunoblots (compare with Fig. 3a, D lanes). Taken together, these results confirmed the 12- and 14-kDa proteins as the HIV-2 and SIV_{MAC} virally encoded X proteins.

To evaluate whether the SIV_{MAC} X protein synthesized in *E. coli* could be of diagnostic value, we examined a limited number of patient and normal control sera for reactivity with this protein (Fig. 4). Four sera from healthy West African HIV-2-infected individuals (Fig. 4a to d), four sera from acquired immunodeficiency syndrome patients seropositive for HIV-1 (Fig. 4e to h), and two healthy control sera (Fig. 4i and j) were tested side by side for reactivity to the 50-kDa TrpE/X protein (Fig. 4, lanes 2), the 15-kDa ΔTrpE/X protein (Fig. 4, lanes 3), and lysates of bacteria transformed with the nonrecombinant pATH vector (Fig. 4, lanes 1). Out of four HIV-2 antibody-positive sera tested, two contained antibodies reactive with both the 50-kDa TrpE/X and 15-kDa ΔTrpE/X proteins (Fig. 4a and c). Four HIV-1-positive sera and two HIV-negative control sera did not react with these X-specific fusion proteins. Bands corresponding to bacterial proteins other than the X fusion proteins were also observed, particularly with the West African sera. However, these were easily distinguishable from X-specific reactivities by their sizes and occurrence in the control antigen preparations.

In summary, the data demonstrate that HIV-2 and SIV_{MAC} viruses encode a novel retroviral protein, termed X, which is expressed in virus cultures in vitro and in naturally infected humans. The molecular mass of this protein, 12 to 14 kDa, approximates that calculated from the deduced amino acid sequence, although the actual structure and organization of the X gene and its protein and the mode of X gene transcription in HIV-2 and SIV_{MAC} viruses cannot be predicted. Similarly unknown at the present time is the function of the X protein in the life cycle of HIV-2 and SIV_{MAC}. The high degree of sequence conservation suggests a significant function for X in both viruses. The fact that HIV-1 lacks the X gene entirely, yet causes disease in infected individuals, implies that X gene expression is not required for induction of cytopathicity in vitro or in vivo. A search for X-related protein sequences in the National Biomedical Research Foundation protein data bank identified several proline-rich regions, present in retroviral proteins, adenovirus, papillomavirus, human collagen, and a group of human phosphoproteins, as sharing homology with the carboxy terminus of the X gene. In addition, a probable nuclear antigen of Epstein-Barr virus (2) showed 10 of 14 conserved amino acids with a region of X just 5' of the

polyproline tract (GRG[H/R]G[P/R]GG[W/G]RPG[P/A]P). The significance of these homologies is under study.

The presence of antibodies specific for X in some HIV-2-infected individuals demonstrates both its immunogenicity and in vivo expression. It will be important to determine if X-specific reagents will be generally useful in distinguishing between HIV-2 and HIV-1 infections, whether expression of X in vivo influences viral pathogenesis and clinical outcome, and whether titers of X-specific antibodies will provide prognostically important clinical information. The availability of X-specific reagents as described in this report should facilitate experiments addressing these questions.

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