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26. We thank L. R. Sykes and E. R. Cook for reviews and L. O. White for field assistance. This work was supported by NSF grants EAR 85-19030 and EAR 87-07967 and U.S. Geological Survey grant 14-08-0001-G1329 to G.C.J. and P.R.S. and U.S. Geological Survey grants 14-08-001-G1098 and G1370 to K.E.S. Lamont-Doherty Geological Observatory contribution No. 4326; California Institute of Technology Geological and Planetary Sciences Contribution No. 4644.

16 March 1988; accepted 31 May 1988

Isolation and Characterization of a Novel Protein (X-ORF Product) from SIV and HIV-2

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A protein designated p14 was purified from a simian immunodeficiency virus (SIV_{Mne}) and was shown by amino acid sequence analysis to be nearly identical to the predicted translational product of a unique open reading frame (X-ORF) in the nucleotide sequences of SIV_{mac} and human immunodeficiency virus type 2 (HIV-2). Thus the X-ORF is proven to be a new retroviral gene. The p14 is present in SIV_{Mne} in molar amounts equivalent to those of the *gag* proteins. This is the first example of a retrovirus that contains a substantial quantity of a viral protein that is not a product of the *gag*, *pro*, *pol*, or *env* genes. SIV p14 and its homolog in HIV-2 may function as nucleic acid binding proteins since purified p14 binds to single-stranded nucleic acids in vitro. Antisera to the purified protein detected p14 in SIV_{Mne}, SIV_{mac}, and a homologous protein (16 kilodaltons) in HIV-2 but did not react with HIV-1. Diagnostic procedures based on this novel protein will distinguish between HIV-1 and HIV-2.

SIMIAN IMMUNODEFICIENCY VIRUSES (SIVs) cause a fatal disease (in susceptible primate species) with symptoms (*1, 2*) similar to those associated with human AIDS, which is caused by human immunodeficiency viruses type 1 (HIV-1) and type 2 (HIV-2). Strains of SIV were originally isolated from rhesus monkeys (*Macaca mulatta*) with immunodeficiency or lymphoma (SIV_{mac}) (*3*), and subsequently from asymptomatic mangabey monkeys (SIV_{SMM}, SIV_{SMLV}, and SIV_{Delta}) (*4*), and from a *Macaca nemestrina* with lymphoma (SIV_{Mne}) (*5*). A strain of SIV originally thought to be obtained from African green monkeys ($STLV\text{-}III_{agm}$) (*6*) has since been shown to be SIV_{mac} (*7*). SIV strains are closely related to each other (greater than 90% identity) (*8, 9*) and also partially related to HIV-1 (40% nucleotide sequence identity) but are more closely related to HIV-2 (75% overall nucleotide sequence identity) (*8*).

The genomic organizations of HIV-1 (*10*), HIV-2 (*11*), and SIV (*8*) are very similar; each contains open reading frames (ORFs) designated *gag*, *pol*, *env*, Q, R, trs, tat, and F. However, HIV-2 and SIV contain an ORF designated X that is not found in HIV-1 (*8, 11*). The X-ORF is located in the central region of the genome between the *pol*-ORF and the *env*-ORF. Here we report the isolation and molecular characterization of a protein from SIV_{Mne} designated p14 and show by amino acid sequence analysis that it is the product of the X-ORF.

A single-cell clone of Hut-78 cells infected with SIV_{Mne} (clone E11S) was grown, and virus was purified by sucrose density gradient centrifugation (*5*). Viral proteins were purified by reversed-phase high-pressure liquid chromatography (RP-HPLC) and characterized by NH_2- and COOH-terminal amino acid sequence analysis (*12*). Our earlier analysis showed that SIV_{Mne} proteins designated p28, p16, p8, p6, p2, and p1 were proteolytic cleavage products of the viral *gag* precursor ($Pr60^{gag}$), which has the following complete structure: p16-p28-p2-p8-p1-p6 (*9*). We also reported a protein (designated p14) (*5*) that did not appear to be a *gag* protein (*9*) but was of viral origin since macaques infected with SIV_{Mne} raised readily detectable antibodies to the protein (*2, 9*).

Partially purified SIV_{Mne} p14 (*9*) was rechromatographed by RP-HPLC to give a homogeneous preparation as shown by SDS–polyacrylamide gel electrophoresis (PAGE) analysis (Fig. 1A, lane 2). Purified p14 was inert to Edman degradation (gas-phase sequencer), which suggested that it had a derivatized NH_2-terminal residue (blocked NH_2-terminus). To obtain amino acid sequence information for identification of p14, we digested the protein with trypsin and purified peptides (Fig. 2A, a to l) for analysis to determine amino acid compositions and sequences. The determined amino acid sequences and compositions were compared with the translated proviral DNA sequence of SIV_{mac} (*8*) and HIV-2 (*11*) and found to be highly homologous to predicted sequences located in the X-ORF of each virus. The SIV_{Mne} p14 peptides (Fig. 2B) align with residues predicted by the X-ORF of HIV-2 starting at position 2 and continue through position 112 except that peptides corresponding to predicted residues 69 through 70 and 85 through 88 were not isolated. Of the 105 amino acid residues of SIV_{Mne} p14 that were determined by analysis

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of purified peptides, 90 were identical to predicted residues in the HIV-2 X-ORF (86% identity) and 103 were found to be identical to the predicted residues in SIV_{mac} X-ORF (98% identity). The amino acid sequence of SIV_{Mne} p14 differs from the sequence predicted by the SIV_{mac} X-ORF in that the SIV_{Mne} protein does not contain aspartic acid at position 3 and has valine substituted for threonine at position 67. In addition, the mature p14 protein has undergone modifications resulting in removal of the initiator methionine and addition of a blocking group to the newly formed NH_2-terminal group. The nature of the modifying group remains to be determined. The COOH-terminal residues of SIV_{Mne} p14 were determined by the rate of release of residues by digestion with carboxypeptidase-P [2 min, Ala (0.30), Leu (0.22); 20 min, Ala (0.40), Leu (0.47)]. These results are consistent with a COOH-terminal amino acid sequence of -Leu-Ala-OH and are in agreement with the sequence deduced from

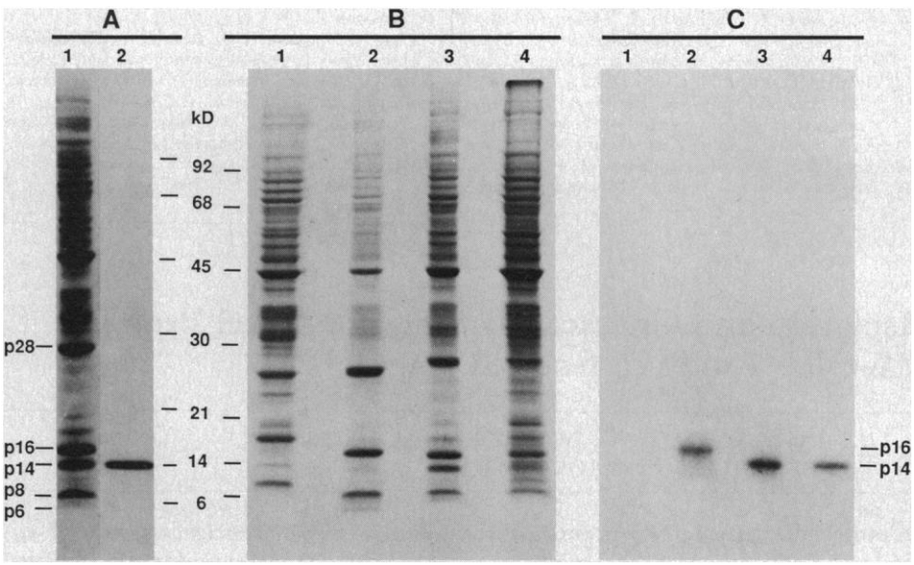

Fig. 1. SDS-PAGE showing protein products of the X-ORFs in SIV_{Mne}, SIV_{mac}, and HIV-2 and the purified protein (p14) obtained from SIV_{Mne}. (**A**) A Coomassie blue–stained gel of SIV_{Mne} (lane 1) and purified p14 (lane 2). Viral *gag* proteins (p28, p16, p8, and p6) are identified in lane 1 (*9*). (**B**) A Coomassie blue–stained gel of HIV-1 (lane 1), HIV-2 (lane 2), SIV_{Mne} (lane 3), and SIV_{mac} (lane 4). (**C**) The results of immunoblot analysis of separated viral proteins [SDS-PAGE identical to that in (B)] with rabbit antiserum to purified SIV_{Mne} p14. After SDS-PAGE, proteins were transferred to nitrocellulose and probed with antiserum at 1 to 500 dilution. Antigen-antibody complexes were detected by radioautography after reaction with ^{125}I-labeled staphylococcal protein A (*19*). Approximately 50 μg of total protein was applied to each viral lane (B) and (C).

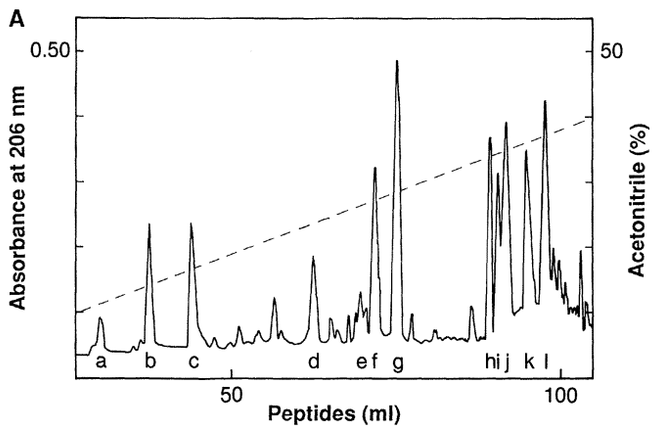

Fig. 2. Amino acid sequences of tryptic peptides from p14 and alignment with the protein predicted by the HIV-2 X-ORF. (**A**) Purification of tryptic peptides. Purified p14 (Fig. 1, lane 2) (200 μg) was dissolved in 0.5 ml of 0.1*M* tris-HCl (*p*H 7.4) and 20 μg of trypsin (Cooper Biomedical) added. The digestion was continued for 8 hours and stopped by adding trifluoroacetic acid (TFA) to *p*H 2.0. Peptides were separated by RP-HPLC at 1.0 ml/min on μ-Bondapak C18 (3.9 by 300 mm column) at *p*H 2.0 (0.05% TFA) with a linear gradient of acetonitrile (– – –) and detected by ultraviolet absorption (——) at 206 nm. Peaks containing peptides taken for further analysis are indicated by the letters a through l. (**B**) Tryptic peptides of SIV_{Mne} p14 aligned with the amino acid sequence predicted by the X-ORF of HIV-2. Tryptic peptides derived from p14 (as in Fig. 2A) were analyzed for amino acid content (Pico Tag system, Waters) and sequence (gas-phase sequencer, model 470A equipped with model 120A analyzer, Applied Biosystems), and the results were compared to the amino acid sequence predicted by the X-ORF in the nucleotide sequence of HIV-2 (*11*). Peptides are indicated by brackets and dashed lines [– – –]. Arrowheads to the right (--->) indicate the last residue identified by amino acid sequence analysis. Arrows to the left (<---) indicate residues determined by digestion of p14 with carboxypeptidase P. Residues in parentheses and separated by commas

B

```
                                        10
HIV-2   Met-Thr-Asp-Pro-Arg-Glu-Thr-Val-Pro-Pro-Gly-Asn-Ser-Gly-Glu-Glu-Thr-
                 *       *  *
SIV-Mne (X,Thr,Ser,Pro)Arg-Glu-Arg-Ile-Pro-Pro-Gly-Asn-Ser-Gly-Glu-Glu-Thr-
        [-------a--------]       [--------------------l-------------------
                                 [-----------------------k----------------------

                    20                                  30
HIV-2   Ile-Gly-Glu-Ala-Phe-Ala-Trp-Leu-Asn-Arg-Thr-Val-Glu-Ala-Ile-Asn-Arg-
                        *                                   *
SIV-Mne Ile-Gly-Glu-Ala-Phe-Glu-Trp-Leu-Asn-Arg Thr-Val-Glu-Glu-Ile-Asn-Arg
        ------------------------------------>] [-----------b----------->]
        ------------------------------------>]

                            40                                          50
HIV-2   Glu-Ala-Val-Asn-His-Leu-Pro-Arg-Glu-Leu-Ile-Phe-Gln-Val-Trp-Gln-Arg-
SIV-Mne Glu-Ala-Val-Asn-His-Leu-Pro-Arg Glu-Leu-Ile-Phe-Gln-Val-Trp-Gln-Arg
        [-----------c--------------->] [---------------h--------------->]

                                    60
HIV-2   Ser-Trp-Arg-Tyr-Trp-His-Asp-Glu-Gln-Gly-Met-Ser-Glu-Ser-Tyr-Thr-Lys-
              *                                                         *
SIV-Mne Ser-Trp-Glu-Tyr-Trp-His-Asp-Glu-Gln-Gly-Met-Ser-Gln-Ser-Tyr-Val-Lys-
        [-------------------------g------------------------------------->]

            70                                          80
HIV-2   Tyr-Arg-Tyr-Leu-Cys-Ile-Ile-Gln-Lys-Ala-Val-Tyr-Met-His-Val-Arg-Lys-
                    *                                   *  *          *  *
SIV-Mne       Tyr-Leu-Cys-Leu-Ile-Gln-Lys Ala-Leu-Phe-Met-His-Cys-Lys
              [-----------e---->-------] [-----------d----------->]

                        90                                              100
HIV-2   Gly-Cys-Thr-Cys-Leu-Gly-Arg-Gly-His-Gly-Pro-Gly-Gly-Trp-Arg-Pro-Gly-
                          *                                 *
SIV-Mne             Cys-Leu-Gly-Glu-Gly-His-Gly-Ala-Gly-Gly-Trp-Arg-Pro-Gly-
                    [--------------------------f-----------------------------

                                    110
HIV-2   Pro-Pro-Pro-Pro-Pro-Pro-Pro-Gly-Leu-Val-OH
                                        *
SIV-Mne Pro(Pro,Pro,Pro,Pro,Pro,Pro,Gly)Leu-Ala-OH
        -->-----------------------------------]
                                          <-- <--
```

represent residues confirmed by amino acid content of purified peptides. Lowercase letters (a, b, c, and so on) refer to peptides purified in Fig. 2A. Asterisks (*) show where the amino acid sequences differ. The unknown blocking group on the NH_2-terminal end of p14 is indicated by x.

the last two codons of the X-ORFs of HIV-2 and SIV_{mac}. The degree of amino acid sequence identity between the protein predicted by the X-ORF of SIV_{mac} and SIV_{Mne} p14 (98% identities) is greater than the degree of identity found for proteins derived from the *gag* gene (92% identity) (*9*) and also greater than the degree of identity found between the two viruses for a 1.6-kb proviral DNA fragment including the 3′ long terminal repeat (93% identities) (*13*). Thus, it appears that the X-ORF protein is highly conserved among SIV strains.

To estimate the molar amount of p14 present in the viral preparation and to relate this amount to the molar amounts of other known viral proteins, we cut the p28, p16, p14, and p8 bands from the gel shown in Fig. 1A, lane 1, and determined their protein content by amino acid analysis as described (*9, 12*). The results were in agreement with the known amino acid content of each protein and showed that the gel contained the proteins in the following molar ratios, 1.0:1.2:1.1:0.8 (p28:p16:p8:p14). Several preparations of SIV_{Mne} including viruses obtained from an infectious molecular clone (*13*) were examined by visual inspection of bands after SDS-PAGE and found to contain similar ratios of p14 to p16 as shown in Fig. 1A, lane 1.

A polyclonal rabbit antiserum to purified SIV_{Mne} p14 (*9*) was used to detect p14 in SIV_{Mne} and probe for cross-reactive proteins in HIV-1, HIV-2, and SIV_{mac} by immunoblot analysis (Fig. 1C). The serum detected a 14-kD band in SIV_{Mne} (lane 3) and SIV_{mac} (lane 4) and a 16-kD band in HIV-2 (lane 2) but did not appear to react significantly with proteins in HIV-1 (lane 1). The detected antigen in SIV_{mac} has the same mobility in SDS-PAGE as SIV_{Mne} p14, and we conclude that it is the translational product of the SIV_{mac} X-ORF. The detected antigen in HIV-2 (p16) has a slower mobility in SDS-PAGE than the p14s of SIV_{Mne} and SIV_{mac} (Fig. 1C). However, SIV isolates from other primate species appear to have cross-reactive proteins with mobilities more similar to the protein detected in HIV-2 than that in SIV_{Mne}. At present, the observed difference in the SDS-PAGE mobilities is unexplained but could be due to differences in amino acid compositions of the proteins rather than differences in actual molecular weights. In any case, the data support the conclusion that the X-ORF translational products of HIV-2 and SIV_{mac} are found in the purified viruses and that HIV-1 does not contain a similar cross-reactive protein.

To visualize and estimate the amounts of viral proteins in each viral preparation, we stained an SDS-PAGE gel identical to that used for immunoblotting (Fig. 1B). Coomassie blue–stained bands at 14 and 16 kD (p14 and $p16^{gag}$) are readily apparent in SIV_{Mne} (lane 3) and SIV_{mac} (lane 4); however, in HIV-2 (lane 2) the 14-kD band is absent and there is a prominent 16-kD band. The 16-kD band in the HIV-2 lane contains both $p16^{gag}$ (data not shown) and the X-ORF protein. The staining intensities of the major *gag* proteins (HIV-1 p24 and p17, HIV-2 p26, and SIV p28s and p16s) may be taken as relative indicators of the amount of virus applied to each lane. A comparison of the intensities of the bands in the immunoblot analysis (Fig. 1C) to the intensities of the Coomassie blue–stained bands (Fig. 1B) suggests that SIV_{mac} and HIV-2 may contain as much of the translational products from their X-ORFs as is observed for p14 in SIV_{Mne}.

The proved structure and genetic origin of p14 show that the X-ORF functions as a gene in SIV_{Mne}. Since the gene and gene product (p14) were first recognized in SIV_{Mne} (*2, 5, 9*) and appear unique to simian immunodeficiency (and closely related) viruses, we propose the gene be named *sid*.

The amino acid sequences of SIV and HIV-2 *sid* proteins contain conserved cysteine residues in positions 73, 87, and 89 and a histidine residue in position 82 (Fig. 2B). Similar cysteine-histidine motifs are thought to play a role in the nucleic acid binding properties of retroviral (*14*) and other proteins (*15*). Purified p14 was tested for nucleic acid binding activity by the method of fluorescence enhancement, with polyethenoadenine used as a single-stranded polynucleotide template (*16*). Figure 3 shows the titration curve obtained by adding p14 to a solution of polyethenoadenine and the titration results obtained for SIV_{Mne} $p8^{gag}$ nucleocapsid protein and $p28^{gag}$. The data show that p14 and p8 bind to the template and indicate that these proteins are capable of binding to single-stranded RNA. The shape of the titration curve is dependent on the degree and nature of cooperative binding (*17*), which is different for each protein. The capacity of p14 to bind to single-stranded nucleic acids may in part account for its apparent high concentration in the purified virus. However, the fact that p14 is capable of binding to single-stranded nucleic acids suggests that it may function in vivo as a specific RNA binding protein.

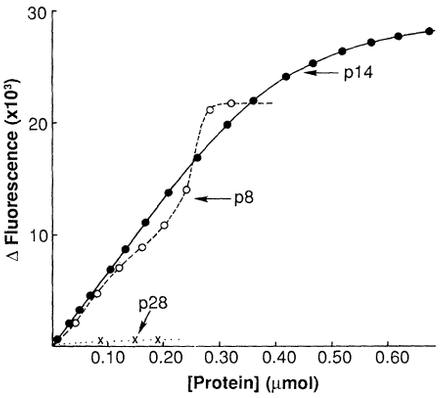

Fig. 3. Purified p14 binds to polyethenoadenylic acid. Purified SIV_{Mne} proteins including p28, p2, p8, p6, and p14 were tested for single-stranded nucleic acid binding activity by the fluorescence enhancement assay with 2.19 μ*M* polyethenoadenine (*16*). Of the viral proteins tested only p14 and p8 produced significant enhancement of fluorescence when added to solutions of polyethenoadenine.

Knowledge of the biological role of the *sid* protein will be important to our understanding of the similarities and differences among the various HIVs and SIVs and may lead to important therapeutic applications. Because the *sid* protein is unique to the HIV-2/SIV group of viruses, diagnostic procedures that use this protein may be developed to unequivocally distinguish between HIV-1, and HIV-2, and strains of SIV.

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20. We are indebted to J. Kern and M. G. Sarngadharan for providing a sample of SDS-disrupted HIV-2. We thank Y. Kim, C. Hixon, R. W. Hill, and W. B. Knott for technical assistance and C. Shawver and C. Rhoderick for help in preparing the manuscript. Research sponsored by the National Cancer Institute, Department of Health and Human Services, under contract N01-CO-74101 with Bionetics Research, Inc.

22 March 1988; accepted 17 May 1988