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Isolation and Partial Characterization of an Unusual Human Immunodeficiency Retrovirus from Two Persons of West-Central African Origin

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An unusual human retrovirus was isolated from two patients with persistent generalized lymphadenopathy who originate from West-Central Africa and are currently residing in Belgium. Although the virus shared a number of the same biological and morphological properties as human immunodeficiency retrovirus type 1 (HIV-1) and HIV-2, significant antigenic differences could be demonstrated. Several of the viral proteins also differed in molecular weight from the corresponding HIV-1 and HIV-2 proteins. Partial chemical cleavage of the most highly conserved viral proteins resulted in patterns which differed from those of HIV-1 and HIV-2. Furthermore, nucleic acid hybridization experiments were capable of discriminating between the virus types. Sequence analysis of the viral U3 region revealed a unique enhancer organization not found in other immunodeficiency viruses. The data indicated that the new isolate is more closely related to HIV-1 than to HIV-2 but clearly differs in a number of important respects.

Until the recent outbreak of acquired immune deficiency syndrome, lentiviruses were a poorly understood group of retroviruses. The discovery of human immunodeficiency virus type 1 (HIV-1) and its identification as the etiological agent responsible for the majority of acquired immune deficiency syndrome cases resulted in an intensive effort to elucidate the genetic structure, biology, and epidemiology of the virus (3, 13, 30, 32). Studies to determine how widely disseminated the virus was eventually led to the discovery of a second related virus, HIV-2, which was found to be capable of inducing the same clinical symptoms in humans as HIV-1 (2, 6). Further investigations to identify possible animal reservoirs of viral infection have to date been unsuccessful, but these efforts have led to the discovery of at least three distinct simian retroviruses (simian immunodeficiency virus [SIV]), SIVagm (29), SIVmac (7, 20), and SIVmnd (38), which are clearly related to the two known HIVs. All these viruses share a tropism for T4 lymphocytes, a Mg^{2+}-dependent reverse transcriptase activity, similar protein profiles, and a similar morphology.

These viruses of human and simian origin have also been shown to be related serologically, particularly with regard to the proteins encoded by their respective *gag* genes (5, 18, 29, 38). Antiserum raised against the *gag* proteins of one virus will cross-react with the *gag* proteins of the others. However, a serological distinction can frequently be made based on the poor cross-reactivity of the envelope proteins of these viruses. Nevertheless, cross-reaction between envelope proteins has also been observed, and in particular, two of the viruses, HIV-2 and SIVmac, appear to be serologically quite closely related (6).

Numerous isolates of HIV-1 and HIV-2 have been obtained from both African and European or American sources. Sequence studies on both viruses have shown that in some regions of each viral genome, the nucleic acid sequence can be quite variable, while in other regions, the sequence is highly conserved (1, 4, 26, 33, 41). Nucleic acid hybridization experiments provide a means by which different virus types can readily be distinguished, particularly if the probes used are derived from conserved regions of the viral genomes. Hybridization can give an indication of the degree to which virus types are related. When performed under stringent conditions, it is possible to make a distinction between virus types even though they appear to be serologically highly related (6).

Epidemiological studies suggest that HIV-1 is widely prevalent in Central Africa, while HIV-2 occurs mainly in West Africa. These two viruses are genetically distinct and widely separated geographically (5, 31). Sporadic but incompletely documented reports suggest that other types of HIVs or highly divergent variants exist but are not yet widely disseminated (10, 28). These reports are based on serological studies which are frequently difficult to interpret because of individual variations in antibody response and because of the serological cross-reactivity which exists between virus types. We now report a novel isolate obtained from two persons of Cameroonian origin who currently reside in Belgium. The virus, which we have designated ANT 70, was partially characterized and exhibited major differences with respect to HIV-1 and HIV-2 reference strains.

MATERIALS AND METHODS

Virus strains. HIV-1 SF4 was originally isolated in the laboratory of J. Levy (23), and HIV-1 (human T-cell lymphotropic virus type IIIB) was originally isolated in the laboratory of R. Gallo (30). HIV-2rod is the prototype HIV-2 strain described by Clavel and co-workers (6). HIV-2 isolate 53 was isolated in this laboratory (39). SIVmac was generously provided by R. Desrosiers (7), and SIVagm (TYO-1) was the kind gift of M. Hayami (29).

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Virus and cell culture. Virus was isolated essentially as previously described (24) by cocultivation of peripheral blood lymphocytes from the patients with phytohemagglutinin-stimulated lymphocytes from healthy donors in RPMI 1640 medium supplemented with 150 U of interleukin-2 per ml, 5 μg of hydrocortisone per ml, and 10% fetal calf serum. Growth of the virus was monitored by antigen capturing (VCA-HIV; Innogenetics), reverse transcriptase assay, immunofluorescence with a high-titer anti-HIV-1 antiserum, and cytopathic effect. Once virus was detected in culture supernatants, the virus was transferred to MT-4 (22) or Molt 4 clone 8 (21) cells by cocultivation. Extensive cytopathic effect was observed in the culture, with syncytium formation and cell death. However, after several weeks, cell growth could again be observed and reverse transcriptase activity and antigen could be detected in the culture supernatant. The resulting cell lines are stably infected and shed virus continuously into the culture medium. Chronically infected cell lines were routinely cultured in RPMI 1640 supplemented with 10% fetal calf serum. These infected cell lines were also adapted for growth in low-serum medium which consisted of RPMI 1640 supplemented with 0.02% fetal calf serum and 1% Nutridoma HU (Boehringer GmbH, Mannheim, Federal Republic of Germany). Virus production was unaffected, and virus pellets obtained from these supernatants contained considerably less contaminating protein.

Gel electrophoresis and protein blotting. Virus was concentrated from the supernatants of infected cultures by centrifugation and was solubilized by treatment with 2% sodium dodecyl sulfate (SDS) and 2% 2-mercaptoethanol. The solubilized proteins were separated on 12.5% polyacrylamide slab gels by the method of Maizel (27). After electrophoresis, the separated proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Federal Republic of Germany) essentially by the procedure described by Dunn (8). Upon completion of the transfer, the membranes were blocked in phosphate-buffered saline containing 0.2% Nonidet P-40 (NP-40) (Sigma Chemical Co., St. Louis, Mo.) and 0.5% casein (E. Merck AG, Darmstadt, Federal Republic of Germany). Immunological detection of viral proteins was accomplished by incubating the membrane for 1.5 h with a 1:200 dilution of an appropriate serum. After washing, the membranes were incubated with a 1:7,500 dilution of goat anti-human immunoglobulin G (IgG)-alkaline phosphatase conjugate (Promega, Leiden, The Netherlands) for 1 h. The membranes were washed extensively, and proteins were visualized by the addition of substrate solution containing 166 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml and 330 μg of Nitro Blue Tetrazolium per ml.

Antigen capturing assays. Either antiserum or ascites fluid was diluted 1:200 in 10 mM Tris–10 mM NaCl (pH 8.5) and used to coat Maxisorp microwell plates (Nunc, Roskilde, Denmark). Coating was done for 1 h at 37°C. The coated plates were then incubated for 1 h with virus-containing culture supernatants which had been treated with NP-40 (0.5%) and diluted 1:5. Unbound antigen was removed by washing five times with phosphate-buffered saline containing 0.05% Tween 20 (Merck). Bound antigen was detected by incubating the plates for 1 h with a high-titer polyclonal anti-HIV-1 IgG preparation coupled to horseradish peroxidase. After the plates were washed to remove unbound conjugate, substrate solution containing 87 μg of 3,3′,5,5′-tetramethylbenzidine (Boehringer) per ml and 0.006% H_2O_2 was added. Color development was stopped after 15 min by the addition of 50 μl of 4 M H_2SO_4, after which the absorbance was read at 450 nm.

Chemical cleavage of viral proteins. Detergent-solubilized lysates of the viruses to be compared were separated in parallel by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was fixed and stained with Coomassie brilliant blue except for a marker lane of HIV-1 protein which was excised and blotted. The blot was incubated with an anti-HIV-1 antiserum to reveal the positions of the viral proteins. Horizontal gel slices containing individual viral proteins were excised from the Coomassie brilliant blue-stained gel, placed in glass tubes, and subjected to chemical cleavage with either CNBr (40 mg/ml in 0.3 N HCl for 3 h) or 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine (BNPS-Skatole, 1 mg/ml in 70% acetic acid–0.1% phenol for 3 h, protected from light). After cleavage, the gel slices were washed and equilibrated for electrophoresis. Blocks corresponding to the lanes in the original gel were excised, rotated 90°, and imbedded on top of a 10 to 20% SDS-polyacrylamide gradient gel. Upon completion of electrophoresis, the gel was electroblotted onto nitrocellulose and incubated with a high-titer anti-HIV-1 antiserum to reveal the spots. Cleavage products with molecular sizes of less than 10 kilodaltons were not detected since they bind poorly to nitrocellulose.

Molecular cloning. Virus was harvested from 1 liter of culture supernatant by centrifugation. The resulting pellet was disrupted in 6 M guanidium chloride, and the RNA was centrifuged through a cushion of 5.5 M CsCl. After centrifugation, the RNA was redissolved, extracted with phenol, and ethanol precipitated. One-fifth of the resulting RNA was used to direct the first step in the oligo(dT)-primed synthesis of cDNA with a commercially available kit according to the instructions of the manufacturer (Amersham International, Amersham, United Kingdom). *Eco*RI linkers were attached, and the size of the cDNA was determined on a 1.2% agarose gel. The region of the gel corresponding to a cDNA length of 800 to 2,000 base pairs (bp) was excised, and the cDNA was eluted and cloned in the vector pUC13. After ligation, the DNA was used to transform competent cells of *Escherichia coli* MC1016 (λ). The resulting colonies were transferred to nylon membrane filters (Pall Biodyne H, Portsmouth, United Kingdom) and screened with a probe containing sequences from the 5′ end of the proviral HIV-1 genome extending to the *Eco*RI site at nucleotide 4229. The hybridization was performed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–0.2% SDS–250 mg of denatured salmon sperm DNA per ml overnight at 65°C. After hybridization, the filters were washed for 1 h in 2× SSC–0.1% SDS at room temperature, 30 min in 0.1× SSC–0.1% SDS at room temperature, 20 min in 2× SSC–0.1% SDS at 42°C, and 20 min in 0.1× SSC–0.1% SDS at 42°C. The filters were subsequently autoradiographed at −70°C with an intensifying screen. A number of weakly positive colonies were identified which were grown for clone analysis. One insert of approximately 900 bp was subcloned in pUC13 (pnefU3R70) and sequenced by using a commercially available kit according to the instructions of the manufacturer (Amersham International).

Dot-blot hybridizations. Virus was harvested by centrifugation after first determining the reverse transcriptase activity present in the culture supernatant. Pelleted virus was disrupted in 10 mM Tris–10 mM NaCl–10 mM EDTA–0.5% SDS, pH 7.4. Aliquots corresponding to 1×10^6, 1×10^5, and 2.5×10^4 cpm of reverse transcriptase activity were spotted onto Hybond N membranes (Amersham International) and irradiated for 2 min under UV light. Hybridizations were performed under nonstringent conditions (5×

SSC, 25% formamide, 5× Denhardt solution, 10% dextran sulfate, 0.1% SDS, 100 μg of denatured salmon sperm DNA per ml at 42°C for 18 h, followed by four 15-min washes in 0.1× SSC–0.1% SDS at room temperature, T_m − 38°C) and autoradiographed. The filters were subsequently washed twice under stringent conditions (twice for 30 min each in 0.1× SSC–0.1% SDS at 63°C, T_m − 10°C) and again autoradiographed.

RESULTS

Virus isolations. The virus (ANT 70) was first isolated from a 19-year-old woman who was identified as being moderately seropositive (ratio OD/cutoff of 4.5) in an enzyme immunoassay for HIV-1 antibodies (Vironostika, Organon, Turnhout, Belgium). Her serum had a low titer in the indirect immunofluorescent antibody assay for HIV-1 (1:40) and gave faint bands in the HIV-1 Western blot (immunoblot) assay (Du Pont Co., Wilmington, Del.) at p24, gp41, and p53/p65. The woman had elevated serum IgG and IgM levels and a T4/T8 ratio of 0.25 (10% T4/40% T8) and was originally classified as group II according to the Centers for Disease Control classification system but has since progressed to group III. The woman seroconverted in March 1987. Virus was detected after 52 days in culture and was then used to establish a chronically infected cell line in Molt 4 clone 8 cells. Virus (ANT 70 NA) was later isolated from her spouse, who is also positive in the HIV-1 enzyme immunoassay but has a higher titer. Antiserum from the man also reacted weakly with the HIV-1 gp120 on commercial HIV-1 Western blots (Du Pont). The man has a reduced T4/T8 ratio of 0.43 (10% T4/23% T8) and suffers from lymphadenopathy (Centers for Disease Control group III). Virus was detected in the culture supernatant after 18 days and was subsequently transferred to MT-4 cells to establish a chronically infected cell line.

The virus originally isolated from the woman was first recognized as being significantly different from HIV-1 on the basis of its reduced ability to be captured in a modified antigen capturing assay performed on NP-40-treated culture supernatants (details to be published elsewhere). Its drastically reduced ability to be captured indicated that the virus was antigenically significantly different from all the HIV-1 isolates (>150) tested in this system thus far, including HIV-1mal, which is one of the most highly divergent HIV-1 strains described to date (1).

Analysis of viral proteins. The proteins of the new isolate were compared with the viral proteins of HIV-1 and HIV-2 by SDS-polyacrylamide electrophoresis followed by immunoblotting (Fig. 1). Although the protein profile of ANT 70 was similar to those of HIV-1 and HIV-2, reproducible molecular weight differences were particularly evident for the major viral core protein, which was slightly larger than the corresponding protein of HIV-2, and for the reverse transcriptase proteins, which were slightly smaller than those of HIV-1. Protein profiles obtained for the isolates ANT 70 and ANT 70 NA were identical (data not shown). The apparent molecular weights as determined in the electrophoresis system used are summarized in Table 1.

The binding of various anti-HIV antisera to the different isolates was also investigated (Fig. 1). There was a demonstrable cross-reaction of all the different antisera with the *gag* and *pol* proteins of the three virus isolates. In lanes 2 of Fig. 1B and C, it is clear that sera from the carriers of the new isolates also recognized diffuse proteins with molecular sizes of approximately 40 to 44 and 120 kilodaltons which are

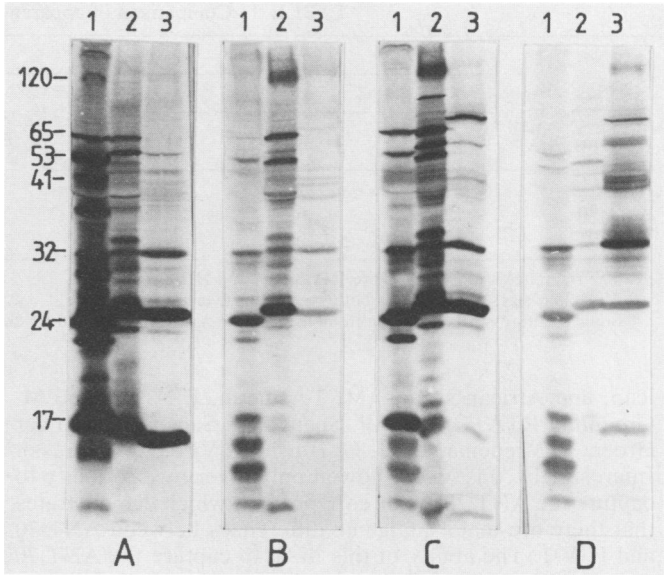

FIG. 1. Visualization of viral proteins and immunological cross-reactions by immunoblotting. Lanes: 1, HIV-1 proteins (strain SF4); 2, ANT 70 proteins; 3, HIV-2 proteins (isolate 53, a laboratory isolate of HIV-2 shown to be nearly indistinguishable from HIV-2rod). (A) Blot incubated with anti-HIV-1 antiserum. (B) Blot incubated with antiserum from the woman from whom ANT 70 was isolated. (C) Blot incubated with antiserum from her partner. (D) Blot incubated with anti-HIV-2 antiserum. A goat anti-human IgG-alkaline phosphatase conjugate was used for detection.

presumably the viral transmembrane protein and the outer membrane protein, respectively. Serum from the man, which was previously shown to have a higher titer in the HIV-1 enzyme immunoassay, also recognized the gp41 protein of HIV-1 (Fig. 1C). Antiserum to HIV-1 or HIV-2 failed to give a detectable reaction with the presumptive gp120 of ANT 70. The anti-HIV-2 serum used had a low titer but cross-reacted with the *gag* proteins of HIV-1 and ANT 70. This serum reacted only with the envelope proteins of HIV-2. On the basis of immunoblots, the isolate ANT 70 appears to be more closely related to HIV-1 than to HIV-2.

Binding of anti-HIV-1 mouse MAbs. To examine further the antigenic relationship between ANT 70 and HIV-1 and HIV-2, monoclonal antibodies (MAbs) which were raised against the p24 and p18 core proteins of HIV-1 strain IIIB were coated onto the wells of microwell plates and tested for their ability to capture the equivalent proteins of HIV-1 SF4, ANT 70, HIV-2rod, and HIV-2 isolate 53 from NP-40-treated culture supernatants (Fig. 2). A high-titer anti-HIV-1 serum which was shown previously to cross-react with all the HIV isolates tested was used to coat control wells to demonstrate that sufficient antigen was present in the lysates to give a maximum signal. Detection was achieved by using a high-titer polyclonal IgG preparation coupled to horseradish peroxidase.

All the MAbs used were able to capture the corresponding antigen of HIV-1 SF4. Four of the MAbs were able to capture the major *gag* protein of ANT 70 but only very weakly. The two MAbs which gave the highest binding (CLB 59 and CLB 21) recognized the same epitope. All other MAbs recognized different epitopes. Two MAbs showed very weak but measurable binding to the major core protein of HIV-2. MAb CLB 14 has been shown to recognize all isolates of HIV-1 tested (>200) including European, Amer-

TABLE 1. Comparison of apparent molecular sizes of viral gene products

Virus[a]	Kilodaltons						
	gag			*pol*		*env*	
	Protein 1	Protein 2	Protein 3	Endo	Reverse transcriptase[b]	Transmembrane protein	Outer membrane protein
HIV-1	12	17[c]	24	31	49, 65	42–45	120
ANT 70	12	16.5	24.8	31	48.5, 62	42–45	120
HIV-2	12	16	24.3	31	51, 66	42–45	120

[a] The reference strains used were HIV-1 SF4 and HIV-2rod.
[b] Molecular sizes are given for both species of reverse transcriptase.
[c] Some strain-to-strain variation in molecular size has been observed for this protein.

ican, and African strains (M. Tersmette, I. N. Winkel, M. Groenink, R. A. Gruters, P. Spence, E. Saman, G. van der Groen, F. Miedema, and J. G. Huisman, Virology, in press). Interestingly, this MAb showed only a very weak ability to capture the ANT 70 major core protein, which demonstrates that there are major antigenic differences between ANT 70 and HIV-1. The ability of this MAb to capture the ANT 70 core protein was of the same magnitude as its ability to capture the corresponding protein of HIV-2.

Binding of antisera to viral antigens. Given the antigenic differences between the virus isolates, it would be predicted that enzyme immunoassays based on antigens of each of the isolates would preferentially bind antibodies made specifically in response to these antigens. HIV-1, HIV-2, and ANT 70 were harvested from the supernatants of infected cultures and solubilized with 1% SDS, and the protein concentration was determined. The solubilized viral protein was diluted in 10 mM Tris–10 mM NaCl–10 mM NaN_3 (pH 8.5) to give a final concentration of 0.5 μg/ml and was used to coat microwell plates. Sera from HIV-1-, HIV-2-, and ANT 70-infected individuals were tested for their ability to bind to wells of microwell plates coated with lysates of the three viral isolates (Fig. 3). In each case, the different sera gave the highest titers when titrated on plates coated with their respective virus lysates.

Partial cleavage of viral proteins. Partial cleavage maps were made of a number of the most highly conserved viral *gag* and *pol* gene products of each virus isolate. Inspection of the published amino acid sequences of HIV-1 and HIV-2 as well as SIVs revealed that the positions of methionine residues, and to an even greater extent tryptophan residues, are highly conserved. These residues are subject to cleavage by CNBr and BNPS-Skatole, respectively (11, 17). Chemical cleavage was performed on polyacrylamide gel slices containing HIV-1 SF4, ANT 70, HIV-2rod, and HIV-2 isolate 53 proteins, and the resulting patterns were compared (Fig. 4). CNBr cleavage of the major core proteins (p24) and reverse transcriptase of HIV-1, HIV-2, and ANT 70 resulted in distinctive patterns which indicated that there are differences between the isolates in the positions or numbers or both of methionine residues. The patterns obtained for HIV-2rod and HIV-2 isolate 53 proteins were, however,

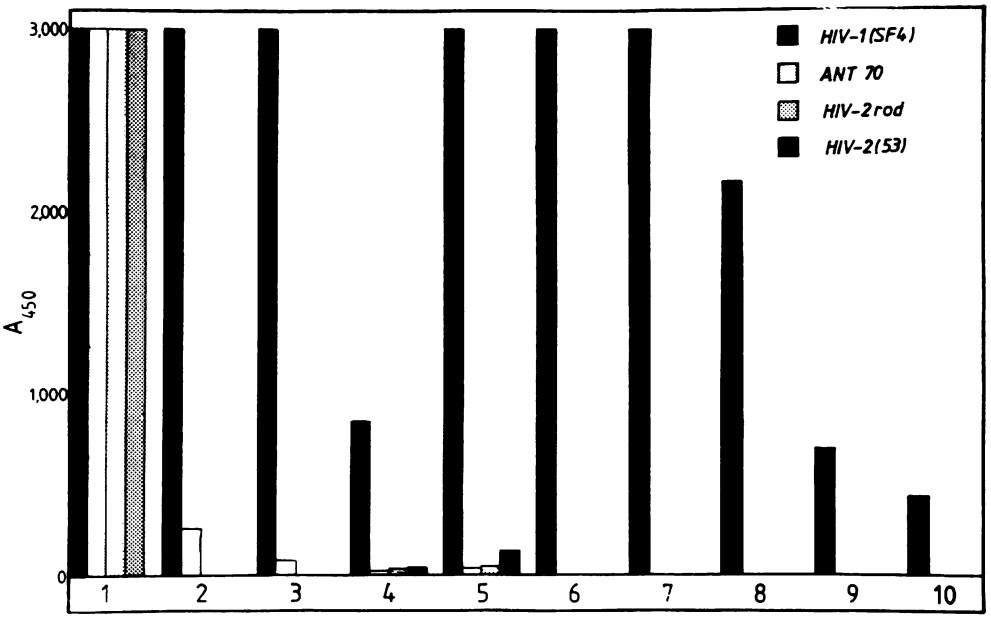

FIG. 2. Antigen capturing with anti-HIV-1 p24 and p18 mouse MAbs. Ascites fluid was used to coat the wells of microwell plates. Virus-containing culture supernatants treated with NP-40 (0.5%) and diluted 1 to 5 were used as a source of antigen. Wells were coated as follows: 1, high-titer polyclonal human anti-HIV-1 antiserum; 2, MAb CLB 59 (anti-p24); 3, MAb CLB 21 (anti-p24); 4, MAb CLB 64 (anti-p24); 5, MAb CLB 14 (anti-p24); 6, MAb CLB 16 (anti-p24); 7, MAb CLB 47 (anti-p24); 8, MAb CLB 13.6 (anti-p18); 9, MAb CLB 19.7 (anti-p24); 10, MAb CLB 13.4 (anti-p18).

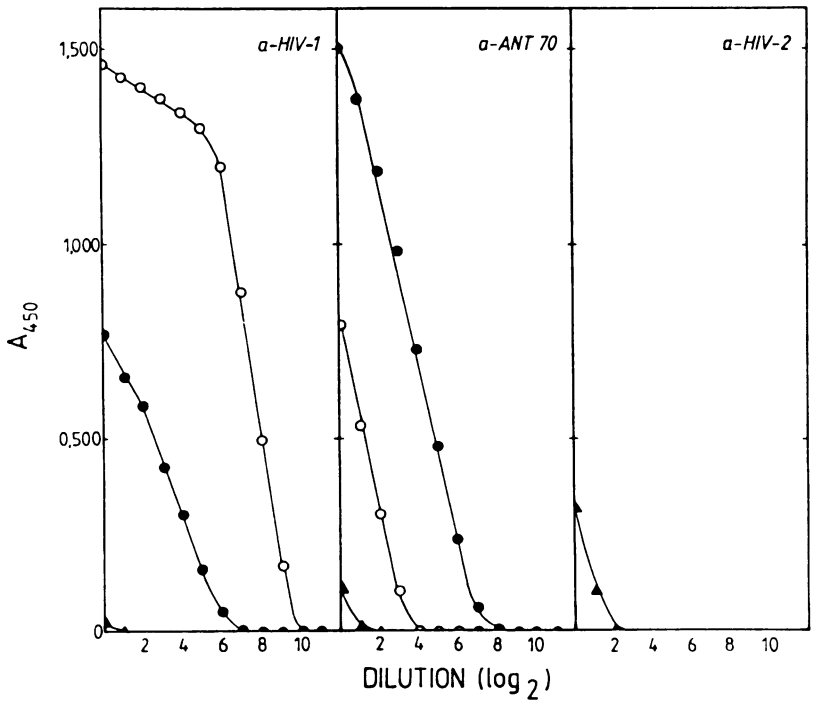

FIG. 3. Titration of anti-HIV antisera on microwell plates coated with HIV-1, ANT 70, and HIV-2 virus lysates. Virus was concentrated and inactivated by detergent treatment. Protein concentration was adjusted to 0.5 μg/ml in 10 mM Tris (pH 8.5)–10 mM NaCl–10 mM NaN_3. Wells were coated with HIV-1 SF4 lysate (○), ANT 70 lysate (●), and HIV-2 (isolate 53) lysate (▲). (A) Titration of anti-HIV-1 antiserum. (B) Titration of anti-ANT 70 antiserum from the partner of the woman from whom ANT 70 was isolated. (C) Titration of anti-HIV-2 antiserum. Antisera were titrated in twofold dilutions beginning at a dilution of 1:100.

identical. Strikingly similar cleavage patterns were obtained when the major *gag* proteins were cleaved at tryptophan residues, which indicates that these are conserved in terms of number and position. Differences were observed in the apparent molecular weights of the central spot in each pattern. The patterns obtained when reverse transcriptase proteins were cleaved with BNPS-Skatole were all different except for HIV-2rod and HIV-2 isolate 53.

Hybridization of subgenomic probes to viral RNA. ANT 70 cDNA clones were isolated by reverse transcription of viral RNA with an oligo(dT) primer. One cDNA clone contained an insert of approximately 900 bp corresponding to the 3′ end of the ANT 70 viral RNA and included the *nef* gene and the 3′ U3 and R regions. Dot-blot filters of viral RNA of HIV-1, HIV-2, SIVagm, SIVmac, and ANT 70 were prepared and hybridized to a labeled ANT 70 cDNA *Bgl*II fragment 472 bp long containing a portion of *nef* and extending into the U3 region of the 3′ long terminal repeat (LTR). Hybridizations were also done with the HIV-1 *Pst*I-*Eco*RI restriction fragment corresponding to nucleotides 966 to 4229 (*gag* to *pol*) and a clone of HIV-2rod containing sequences corresponding to nucleotides 3706 to 8569 (*pol* to *env*). Hybridization was performed under nonstringent conditions and was visualized by autoradiography. The filters were subsequently washed under stringent conditions and again autoradiographed. Under nonstringent conditions, crosshybridization was detectable between HIV-1 and ANT 70 (Fig. 5). However, under stringent conditions, the various probes only hybridized to the RNAs of the viruses to which they corresponded. The differences between ANT 70 and HIV-1 are therefore of such a magnitude, even in the *gag-pol* region, that the formation of a stable hybrid is precluded under stringent conditions.

Nucleotide sequence of ANT 70 U3 and R regions. The nucleotide sequence of the insert contained in the plasmid pnefU3R70 was determined and was found to correspond to the 3′ end of the viral genome. The sequences of the U3 and R regions of the viral LTR are shown in Fig. 6. For purposes of comparison, the sequence is aligned with the corresponding sequence found in HIV-1bru (40). The overall sequence homology in this region between ANT 70 and HIV-1bru or HIV-1mal was found to be approximately 70%. The ANT 70 U3 and R regions were 487 and 96 nucleotides long, respectively. The U3 and R regions of HIV-1bru are, by comparison, 453 and 98 nucleotides long. The homology between ANT 70 and HIV-2rod or SIVagm in the corresponding region was less than 60%, and the sequences were difficult to align.

Like all HIVs and SIVs, ANT 70 possesses sequences corresponding to core enhancers I and II except that the sequence of ANT 70 core enhancer II deviates slightly from the consensus sequence GGGACTTTCC. In contrast to all human and simian retroviruses isolated to date, the core enhancer sequences of ANT 70 are separated by an 18-bp insert. The sequence of the insert is highly homologous to the sequence located just upstream of core enhancer II in HIV-1bru between positions −116 and −134 relative to the mRNA cap site. A portion of this sequence exhibits homology to a sequence found in the first intron of the gamma interferon gene (36). Small three-nucleotide and one-nucleotide inserts are located just 5′ to ANT 70 core enhancer II. The ANT 70 sequences between nucleotides −123 and −140 and between nucleotides −139 and −155 show a very high degree of homology (15 of 18) with the insert located between the two core enhancers. The HIV-1 sequences located between positions −109 and −130 which bear a

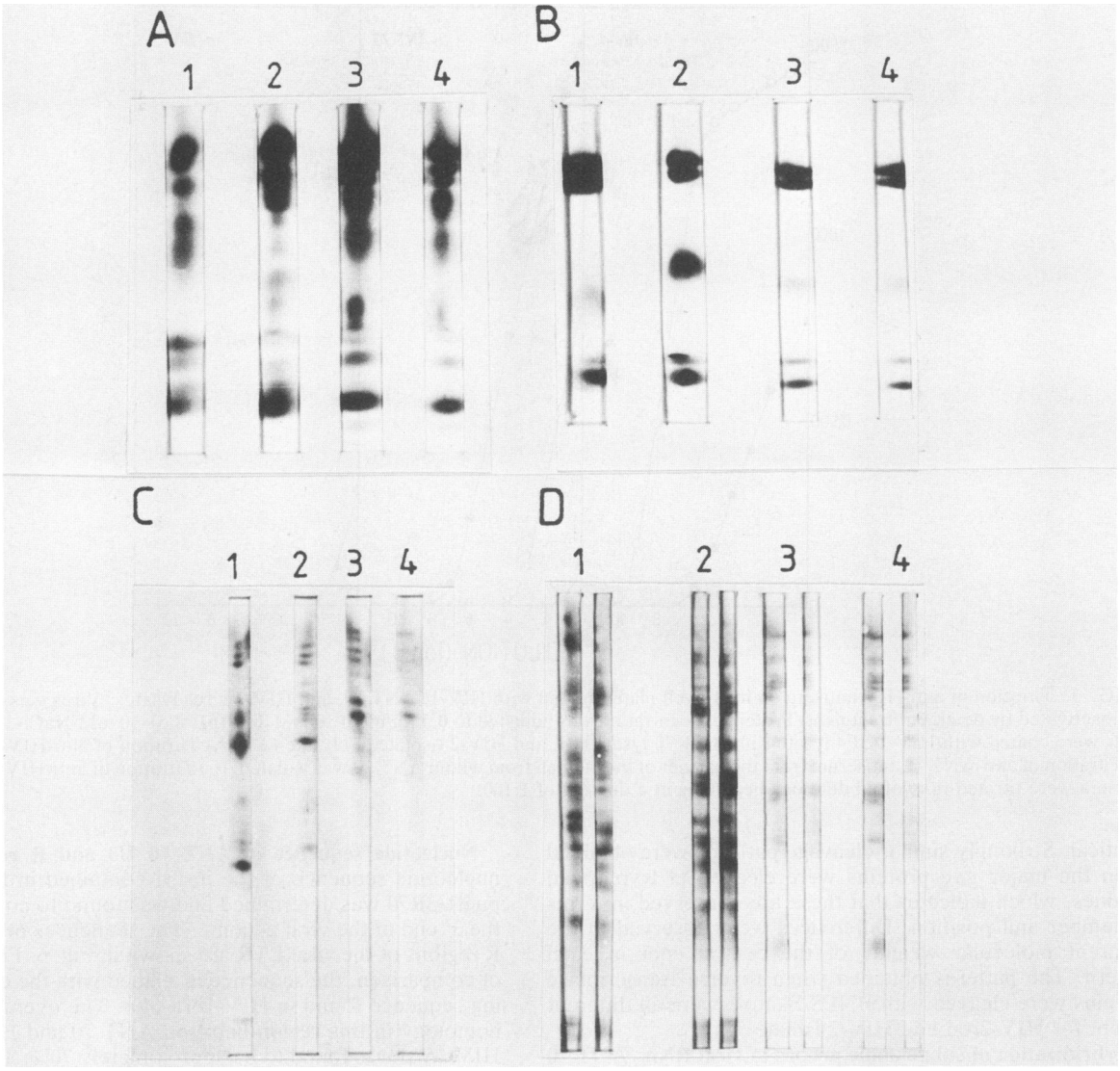

FIG. 4. Comparison of partial cleavage products of viral proteins. Viral lysates were separated by SDS-polyacrylamide gel electrophoresis in parallel lanes. After electrophoresis, the gel was treated as described in Materials and Methods. (A) CNBr cleavage of viral p24 core proteins. (B) BNPS-Skatole cleavage of viral p24 core proteins. (C) CNBr cleavage of the smaller viral reverse transcriptase species. (D) BNPS-Skatole cleavage of both small and large forms of viral reverse transcriptase proteins. Lanes: 1, HIV-1 SF4; 2, ANT 70; 3, HIV-2rod; 4, HIV-2 (isolate 53, a laboratory isolate).

resemblance to the sequence found in the first intron of the gamma interferon gene are largely preserved in ANT 70. Furthermore, ANT 70 contains additional insertions between nucleotides −151 and −165 which create a sequence which is highly homologous to a sequence found in the 5′-flanking regions of interleukin-2 and other T-cell-specific genes (12, 19):

AG/CAAAG/CGAGGAAAAACTG consensus
−163 A G AA−G GACTAAAAACTG −147 ANT 70

The HIV-1 U3 region between −159 and −173 has been identified as a possible negative regulatory region, and protection by cellular proteins has been observed in DNase I footprinting experiments (15). Little homology was observed in the corresponding ANT 70 sequence in this region. The HIV-1 U3 domain situated between nucleotides −176 and −278 has been shown to contain elements which amplify the transcription of linked reporter genes in response to stimuli such as phytohemagglutinin, phorbol 12-myristate 13-acetate, or calcium ionophore (35, 37). Furthermore, a protein present in activated T cells, the nuclear factor of activated T cells or NFAT-1, has been shown by protection experiments to bind the HIV-1 LTR in the region located between nucleotides −216 and −254 (34). A comparison of this region with the corresponding region of the ANT 70 LTR revealed only a modest degree of homology (50%) between the two viruses.

A negative regulatory domain has been shown to be present in the HIV-1 LTR between nucleotides −278 and −340 which acts to down-regulate LTR-directed transcription in response to phytohemagglutinin and phorbol 12-myristate 13-acetate as well as the HIV-1 *tat* protein. Again,

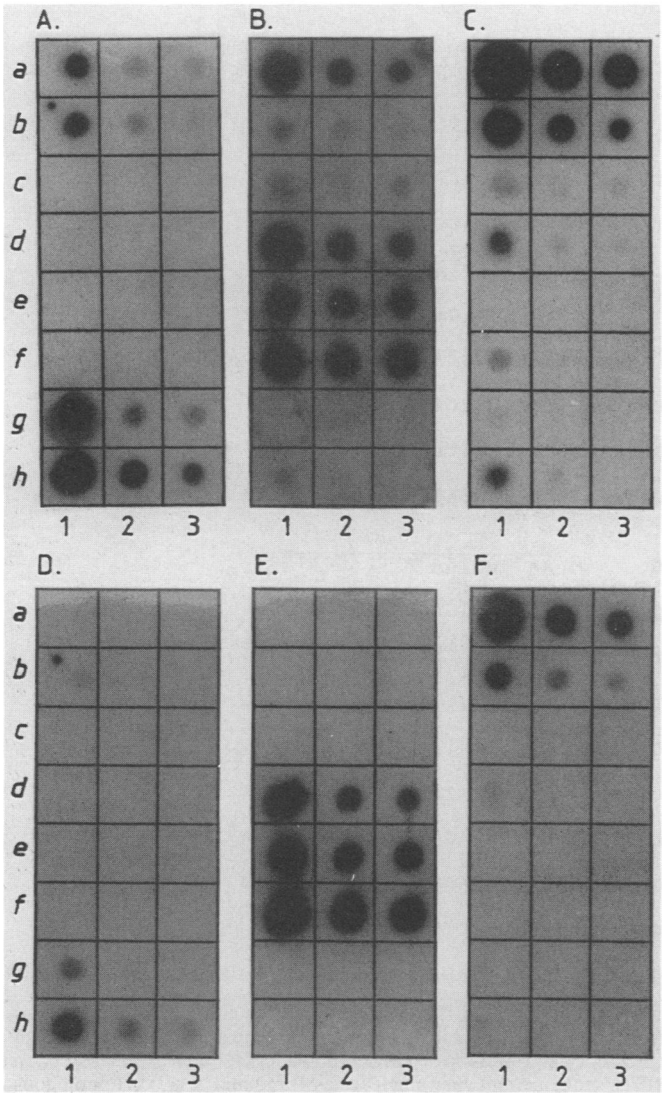

FIG. 5. Hybridization of viral genomic RNA with subgenomic DNA probes. Virus was harvested from culture supernatants, spotted onto Hybond N membranes, and irradiated for 2 min under UV light as described in Materials and Methods. Viruses were spotted in quantities corresponding to 1×10^6 cpm (column 1), 1×10^5 cpm (column 2), and 2.5×10^4 cpm (column 3) of reverse transcriptase activity. The viruses tested were ANT 70 NA (partner) (row a), ANT 70 (row b), SIVagm (row c), SIVmac (row d), HIV-2 isolate 53 (row e), HIV-2rod (row f), HIV-1 SF4 (row g), and HIV-1 (human T-cell lymphotropic virus type IIIB) (row h). The filters are shown for both nonstringent (A, B, and C) as well as stringent (D, E, F) conditions. Hybridizations were done with probes derived from HIV-1 (A and D), HIV-2 (B and E), and ANT 70 (C and F) as described in the text.

the homology between HIV-1 and the corresponding ANT 70 sequences in this region was modest, particularly in the 3′ half of this domain.

The ANT 70 LTR contains G+C-rich sequences 3′ to core enhancer I which are presumably binding sites for the transcription factor Sp1. Little homology was observed between HIV-1bru and ANT 70 for the sites Sp1-3 and Sp1-2. The TATA box was found beginning at nucleotide −27, surrounded by two imperfect repeats (IRs).

The ANT 70 R region possessed sequences capable of forming a stable stem-loop structure with the sequence CCCGGG located in the loop (Fig. 7). However, the repeats CTCTCTGG (nucleotides +5 to +12 and +37 to +44) normally found in the HIV-1 transactivation-responsive (TAR) region and which are highly conserved among HIV-1 isolates were partially deleted or altered in ANT 70. Sequences have been identified in the stem of the HIV-1 TAR region which appear to be essential for protein binding and transactivation (14). These sequences include at least a portion of the repeat located between nucleotides +5 and +12. The nucleotides TCT are deleted from the first CTCTCTGG repeat in the ANT 70 sequence. The insertion of three nucleotides (GAG, +13 to +15) restored the alignment between the two sequences. Corresponding base changes were also found between positions +46 and +53 which maintain the stem structure.

DISCUSSION

A novel HIV was isolated from two individuals which differs significantly from other isolates in its immunological and biochemical properties as well as in the organization of the viral regulatory region. The virus was originally recognized as being different as a result of its drastically reduced ability to be captured in an antigen capturing assay which identifies HIV-1 isolates based on the presence of a highly conserved structural epitope on the viral p24 core protein. Nevertheless, the new isolate possesses the full complement of retroviral proteins and a Mg^{2+}-dependent reverse transcriptase and has a morphology typical of HIV-1 and -2 (data not shown).

The proteins of the viruses isolated from the two individuals were indistinguishable from each other but were readily distinguishable from other HIV-1 and HIV-2 strains on the basis of the apparent molecular weights of both the major *gag* proteins as well as the two reverse transcriptase species. Serological cross-reaction was also demonstrated between HIV-1, HIV-2, and ANT 70, indicating that these proteins are related. While the serological results showed that ANT 70 is more closely related to HIV-1 than to HIV-2, major antigenic differences were apparent in the antigen capturing experiments with mouse MAbs raised against the p24 and p18 proteins of HIV-1 (human T-cell lymphotropic virus type IIIB).

The experiment in which antisera were evaluated for their ability to bind to plates coated with lysates of HIV-1, HIV-2, and ANT 70 also illustrated the antigenic differences between the viruses and demonstrated that while antibodies to ANT 70 could be detected on HIV-1-coated plates, the sensitivity was much less than on plates coated with ANT 70 lysate. HIV-1, HIV-2, and ANT 70 could be further differentiated on the basis of partial cleavage profiles of the major core protein and reverse transcriptase species and by nucleic acid hybridization.

A determination of the sequences of the U3 and R regions of the ANT 70 LTR revealed an overall homology of only 70% compared with HIV-1bru or HIV-1mal. By comparison, HIV-1mal, one of the most highly divergent HIV-1 strains yet described (1), shares approximately 85% homology with HIV-1bru in the same region.

One of the most surprising observations to emerge from the sequence analysis was the presence of an 18-bp insert situated between the two core enhancers. This suggests a possible functional role for the insert in transcriptional activation. Two other IRs of this same 18-nucleotide sequence were found at two other positions 5′ to ANT 70 core enhancer II:

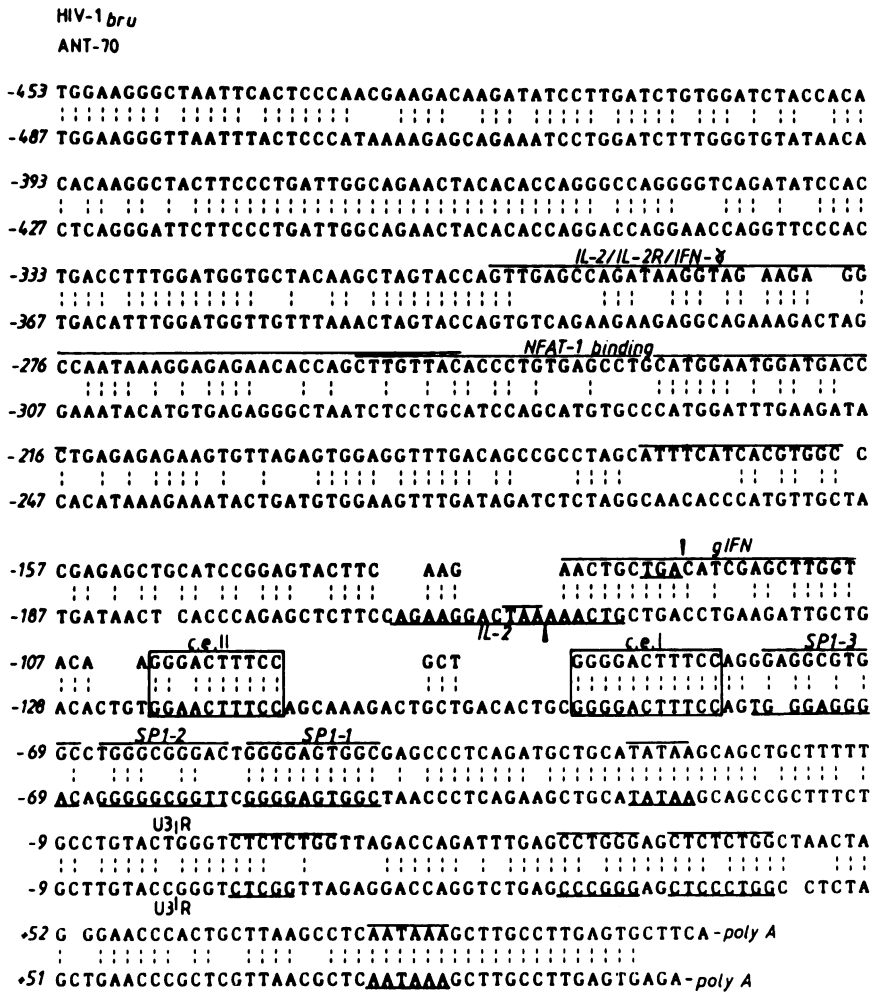

FIG. 6. Sequence of the ANT 70 U3 and R regions and a comparison of the homology with HIV-1bru. The core enhancer sequences are enclosed in boxes for purposes of orientation. Regions of HIV-1 which have proven or putative function are indicated. Other regions of importance are also indicated with horizontal lines (TATA box, CTCTCTGG repeats, TAR loop sequence, polyadenylation signal, stop codon for the *nef* coding sequence, and a negative regulatory region in the HIV-1 sequence between nucleotides −159 and −173). Homologous nucleotides in the HIV-1bru and ANT 70 sequences are indicated by vertical lines. IL-2, Interleukin-2; IFN, interferon.

IR-3 −155 TAAAAACTGCTGAC-CTG −138
IR-2 −140 TGAAGATTGCTGACACTG −123
IR-1 −109 CAAAGACTGCTGACACTG −92 (insert)

The 3′ end of IR-2 is provided by the three-nucleotide insertion at positions −123 to −125 as well as the one-nucleotide insertion at −129.

Additional insertions between −152 and −164 create a sequence found in T-cell-specific genes which overlaps the 5′ end of IR-3. Isolated enhancer sequences which are bound by transcriptional factors utilized in activated T cells must frequently be present in two or more copies to confer T-cell-specific function and inducibility upon linked genes (9, 25, 34). The repetition of the IR sequences may therefore be significant. One highly homologous copy of this sequence is present in the HIV-1 LTR between nucleotides −116 and −134. Although no specific function has yet been ascribed to this sequence, we assume there must be advantages inherent not only in retaining the sequence but in triplicating it.

The physical separation of the two core enhancers is not expected to affect their function to any appreciable extent since it has been shown that they function independently and that the close proximity of these two sequences which is normally observed does not positively or negatively influence their ability to bind transcriptional factors (16). Nevertheless, these same authors also present evidence which suggests that the HIV-1 enhancer element is occupied by only one protein at a time. It is possible that the inserted sequences between the two motifs in the ANT 70 LTR would allow multiple protein binding. Furthermore, the HIV-1 3G sequence was demonstrated to be the stronger of the two when incubated with extracts from phorbol 12-myristate 13-acetate-treated cells. In the ANT 70 LTR, the 3G sequence contains a point mutation, thereby creating a 2G motif. The consequence of this mutation for the binding of transcription factors is as yet unknown. The enhancer region of the ANT 70 U3 region thus contains an array of proven and putative enhancer sequences. The functional relationship between these sequence elements and the core enhancers and their role in transcriptional activation is currently under investigation.

Analysis of the ANT 70 R-region sequences between +1 and +59 revealed that a stem-loop structure can be formed

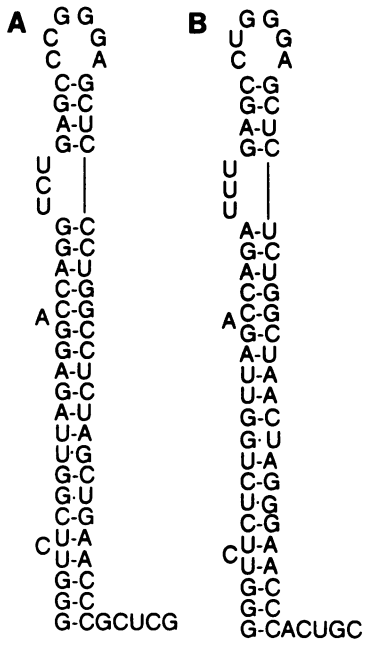

FIG. 7. Stem-loop structure of the ANT 70 TAR region. Structures are shown for ANT 70 (A) and for HIV-1bru (B).

which in many respects closely resembles the stem-loop structure important for transactivation of HIV-1. One very striking difference, however, is the three-nucleotide deletion in the sequence CTCTCTGG normally found at nucleotides +5 to +12 which is highly conserved among HIV-1 isolates. This sequence is evidently a recognition sequence for a cellular protein, referred to as UBP-1, which is required for transactivation and which shows protection over both the TATA and TAR regions in DNase I footprinting experiments (14, 42). In these experiments, the introduction of point mutations in this sequence (CTCTCTGG → CTCGCGGG) resulted in the loss of binding between −42 and +28. It will therefore be of interest to determine to what extent this sequence and surrounding sequences are protected in similar experiments with ANT 70. It has been suggested that the second direct repeat found at nucleotides +37 to +44 is likely to be a degenerate recognition site for UBP-1 (14). This site in the ANT 70 R region also contains a point mutation relative to the HIV-1 sequence; however, the significance of this change remains to be investigated.

In conclusion, although a clear relationship exists between ANT 70 and HIV-1, the new isolate is easily distinguishable at virtually every level of analysis. Since HIV-1 has been so extensively studied, it will be informative to investigate the many differences found in the ANT 70 genome. Furthermore, we expect that ANT 70 will be a valuable reference point for tracking HIV evolution.

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