A Novel Proviral Clone of HIV-2: Biological and Phylogenetic Relationship to Other Primate Immunodeficiency Viruses

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Infectious molecular clones of the human immunodeficiency virus type 2 (HIV-2) will be valuable tools for the study of regulatory gene functions and the development of an animal model for the human acquired immunodeficiency syndrome (AIDS). To this end, we have cloned and sequenced a novel HIV-2 isolate, HIV-2_{BEN}. One clone, designated MK6, is infectious for various human T-cell lines and for human and macaque peripheral blood lymphocytes (PBL), allowing molecular studies of HIV-2 infection and replication. Since MK6 is highly cytopathic in MT-2 and Molt-4 clone 8 cells, antiviral agents and neutralizing sera may be tested. Cluster analysis of HIV-1, HIV-2, and simian immunodeficiency virus (SIV) *env* and *gag* genes revealed that HIV-2_{BEN} yielded the earliest node of phylogenetic divergence for all reported HIV-2 sequences. Noise analysis showed that, with the current data, no specification of any branching order can be made among the four groups of primate lentiviruses, HIV-1, HIV-2/SIV_{SMM/MAC}, SIV_{AGM}, and SIV_{MND}. © 1990 Academic Press, Inc.

INTRODUCTION

Like HIV-1 (Barré-Sinoussi *et al.*, 1983), HIV-2 (Clavel *et al.*, 1986a) may cause characteristic AIDS symptoms and fatal immunodeficiency (Clavel *et al.*, 1987), but it has been suggested that HIV-2 often causes less severe disease (Marlink *et al.*, 1988). In rare cases, HIV-2 may also induce a state in which neurological symptoms predominate (Klemm *et al.*, 1988). Phylogenetically HIV-2 is related to SIV isolated from rhesus macaques (*Macaca mulatta*) (Daniel *et al.*, 1985) and sooty mangabeys (*Cercocebus atys*) (Fultz *et al.*, 1986) and it has been suggested that HIV-2 and SIV_{MAC} may have evolved from SIV_{SMM} (Hirsch *et al.*, 1989). In contrast to HIV-1 which is only capable of infecting chimpanzees and gibbons, but without inducing disease (Alter *et al.*, 1984; Morrow *et al.*, 1989), several SIV isolates cause AIDS-like symptoms in macaques (Murphey-Corb *et al.*, 1986; Daniel *et al.*, 1987; McClure *et al.*, 1989). Recently, it has been demonstrated that vaccination can prevent infection in monkeys (Desrosier *et al.*, 1989; Murphey-Corb *et al.*, 1989). Since HIV-2 infects macaques (Dormont *et al.*, 1989; Stahl-Hennig *et al.*, submitted for publication) it could be of value in the establishment of an animal model for human AIDS.

We have isolated a biologically active molecular clone of HIV-2_{BEN} and studied its biological and immu-

nological features in human T-cell lines as well as in macaque and human PBL. We have also started to investigate the infectivity of the molecular clone for macaques in order to establish an animal model for vaccination and therapeutic studies. The complete sequencing of the proviral DNA made it possible to determine the phylogenetic position of HIV-2_{BEN} in relation to the other primate lentiviruses.

MATERIALS AND METHODS

Cloning and nucleotide sequence analysis

The origin, isolation, and restriction mapping of HIV-2_{BEN} has been described elsewhere (Klemm *et al.*, 1988; Schneider *et al.*, in press). A λ phage library was constructed from the genomic DNA isolated from Molt-4 clone 8 cells infected with HIV-2_{BEN}, as described for HIV-2_{NIH} (Franchini *et al.*, 1989). About 3.5×10^6 recombinant plaques were screened ($T_m = -25^\circ$) with the pROD4.8 and pSPE2 inserts (Clavel *et al.*, 1986b) as probes. Six positive clones were plaque purified and analyzed by restriction mapping (Fig. 1). A set of overlapping restriction fragments of MK2 and MK6 was subcloned into the M13mp18 and M13mp19 vectors (Messing and Viera, 1982) and both strands of the HIV-2_{BEN} genome were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The nucleic acid sequence data were submitted to the Los Alamos HIV Sequence Database (G. Myers).

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FIG. 1. Genomic organization (A) and restriction site maps of the provirus (B) and of five HIV-2_{BEN} molecular clones (C). Restriction enzymes are *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hind*III (H), *Hinc*II (Hc), *Kpn*I (K), *Pst*I (P), *Pvu*II (Pv), *Xho*I (X), and *Xba*I (Xb). Cellular DNA sequences are indicated by broken lines. EMBL-3 vector DNA is indicated by dotted lines. Stars indicate restriction site polymorphism among the molecular clones. The clone MK3 contains the same part of the proviral HIV-2_{BEN} genome as MK2.

Transfection of MK6 to human T-cell lines

About 3×10^6 Molt-4 clone 8 or MT-2 cells were transfected with 10 μ g MK6 phage DNA by the DEAE-dextran method (Sompayrac and Danna, 1981; Milman and Herzberg, 1981). The RT assay was performed according to standard procedures (Hoffmann *et al.*, 1985).

Infection of human and macaque PBL

For infection, 1×10^6 PBL were incubated for 1 hr in cell-free supernatant of MK6-transfected Molt-4 clone 8 cells (RT activity 1×10^6 cpm), washed twice with serum-free RPMI1640 (GIBCO-BRL, Karlsruhe, FRG), and cultivated with RPMI 1640 medium supplemented with 20% FCS (Seromed, Berlin, FRG) and 10% IL-2 (Biotest Diagnostics, Dreieich, FRG). Six days after infection an equal number of fresh PBL was added and 4 days later cocultivation with Molt-4 clone 8 cells was begun.

Western blot analysis

For Western blotting (Towbin and Gordon, 1984), virus from cell culture supernatants was pelleted for 1

hr at 80,000 *g*. Protein (10 μ g) from each pellet was separated by electrophoresis on 8–12% continuous gradient polyacrylamide gels and transferred to nitrocellulose filters (Schleicher & Schüll, Dassel, FRG). Blots were developed using serum from a rhesus monkey experimentally infected with HIV-2_{BEN} and a rabbit antiserum against purified SIV_{AGMP24}.

Polymerase chain reaction (PCR)

For PCR analysis (Ou *et al.*, 1988) 1 μ g of cellular DNA from the cell cultures indicated or 0.1 ng MK6 DNA was dispensed into 100 μ l of reaction buffer (67 mM Tris-HCl, pH 8.8; 16.6 mM (NH₄)₂SO₄; 10 mM β -mercaptoethanol; 0.5% DMSO; 0.17 mg/ml BSA; 4 mM MgCl₂); 50 pM of each primer 1 and 2; 2.5 U Taq polymerase (New England Biolabs); 300 μ M dNTP (Pharmacia, Uppsala, Sweden) and subjected to 35 cycles of amplification using a Biomed PCR processor. One cycle consisted of 1.5 min at 95°, 2 min at 46°, and 1.5 min at 70°. The first denaturation step was prolonged to 6.5 min and the 35th extension step, to 7.5 min. DNA samples were separated on a 2% agarose gel.

Sequence analysis of HIV-2_{BEN}

The phylogenetic tree was constructed using the hierarchical cluster method "average linkage." The computations were done with the CLUSTAN software package (D. Wishart) on an IBM 3090. Pairwise Hamming distances (Hamming, 1950) in the purine/pyrimidine space were determined after alignment of the *env* and *gag* nucleotide sequences using the program GAP of the UWCG software package. Since deletions and insertions were excluded from the further computations, a total of 1992 positions of the aligned *env* sequences were analyzed. The height of the shaded area in Fig. 4A refers to the arithmetic average box dimension in Fig. 4B. The exact geometry in the purine/pyrimidine space was determined for the four *env* sequences that are the most diverged (i.e., inhabiting overall the largest Hamming distances in the computed pair-distance matrix) using a method that combines both horizontal and vertical sequence analysis (Eigen *et al.*, 1988). The box dimensions as well as the protrusion lengths are given in exact positions. All sequences except the HIV-2_{BEN} and the HIV-1_{23U} (now called HIV-1_{HAN2}; Sauermann *et al.*, in press) sequences were derived from the Los Alamos HIV Sequence Database (G. Myers).

RESULTS

Molecular cloning and genomic organization of HIV-2_{BEN}

The restriction enzyme pattern of the six HIV-2_{BEN} clones obtained (Fig. 1C) is considerably different from

A **B**

FIG. 2. Biological activity of MK6. (A) RT activity in Molt-4 clone 8 cells transfected with MK6. RT activity is monitored as [³²P]dGMP incorporation using poly(rC):oligo(dG) as the template primer. Inset: Syncytia formation in transfected Molt-4 clone 8 cells. (B) Western blot analysis of virus purified from the cell-free supernatant of Molt-4 clone 8 (lane 1) and MT-2 (lane 2) cells transfected with MK6. In lane 3 protein of the wild-type HIV-2_{BEN} virus was separated; K, virus has been harvested from the supernatant of mock-transfected MT-2 cells with high background RT. Left, a rabbit antiserum to purified SIV_{AGM} p24 and, right, a serum from a rhesus monkey experimentally infected with HIV-2_{BEN} was used.

those of previously analyzed HIV-2 proviruses (Guyader *et al.*, 1987; Zagury *et al.*, 1988; Franchini *et al.*, 1989; Kong *et al.*, 1988; Kühnel *et al.*, 1989). The com-

plete proviral HIV-2_{BEN} sequence of 10,359 nucleotides was obtained from the clones MK2 (bases 1903 to 5857 and 7293 to 10359) and MK6 (bases 1 to 1902, 5858 to 7292, and 9132 to 10359). The 1227 bases of the 3' region of MK2 and MK6 differed only in four bases. In genetic organization, HIV-2_{BEN} (Fig. 1A) is similar to other HIV-2 isolates (Guyader *et al.*, 1987; Zagury *et al.*, 1988; Franchini *et al.*, 1989). A detailed analysis of the HIV-2_{BEN} sequence will be published elsewhere (Kirchhoff *et al.*, submitted for publication).

MK6 is infectious in human T-cell lines

Eleven days after transfection of Molt-4 clone 8 and MT-2 cells with MK6 DNA syncytia formation occurred in both cell lines, and high RT activity was present in the cell culture supernatants (Fig. 2A). The viral proteins gp160, gp120, gp42, p24, and p18 could be detected in the respective culture supernatants (Fig. 2B). Southern blot analysis revealed that for both cell lines the amount of integrated proviral MK6 DNA considerably exceeded that detected after infection of the same cells with the original HIV-2_{BEN} isolate (data not shown). Since the permanent production of MK6 virus readily caused cell death, transfected cultures had to be supplemented with uninfected cells every 2 to 3 days. In one MK6-transfected Molt-4 clone 8 cell culture neither RT activity nor cytopathic effects were observed

TABLE 1

INFECTIVITY OF MK6 FOR HUMAN T-CELL LINES,
AND HUMAN AND MACAQUE PBL

Cell line	Day of maximal RT after infection	Peak RT activity (pmol dGMP incorp. ml ⁻¹)	Maximal activity in uninfected cells
Molt-4 clone 8	9	110.4	0.9
MT-2	10	60.2	15.8
CEM	23	9.4	0.6
HUT-78	37	5.5	0.7
Jurkat	16	3.1	0.6
Human PBL	18	6.8	0.1
Cynomolgus PBL	7 ^a	6.4	0.2
Rhesus PBL	7 ^a	7.0	0.2

Note. Human T-cell lines were infected with 1×10^6 cpm RT activity of MK6 virus as described for the infection of PBL under Materials and Methods. In MT-2 cell supernatants high background RT activity was measured owing to the fact that this cell line carries the HTLV-1 provirus (Harada *et al.*, 1985).

^a Macaque PBL were cocultivated with Molt-4 clone 8 ten days after infection since they were too sensitive for longer independent cultivation. Therefore, the RT values shown may not represent the real peak activity. All infections were repeated two to five times with similar results.

FIG. 3. Detection of proviral sequences by PCR. Above, the amplified DNA sequence of the HIV-2_{BEN} genome is noted. The primer sequences, the radioactively labeled probe, and restriction enzyme recognition sites used are indicated. Middle, agarose gels with amplified DNA fragments and bottom Southern blots of the same gels. (M) Marker (ϕ X174 *Hae*III digested, New England Biolabs Inc., Beverly, USA); lane 1, undigested DNA; 20- μ l aliquots of amplified DNA were digested with *Bam*HI (lane 2), *Hinc*II (lane 3), and *Bam*HI and *Hinc*II together (lane 4).

6 weeks after transfection. However, substantial amounts of proviral DNA were still present after 2 months of culture and the addition of uninfected cells leads to renewed giant cell formation and virus release.

To examine the cell tropism of MK6, Jurkat, CEM-T4, and HUT-78 cells were infected with culture supernatant from transfected Molt-4 clone 8 cells. In these three cell lines MK6 replicated less efficiently than in Molt-4 clone 8 or MT-2 cells (Table 1). In contrast to infected MT-2 and Molt-4 clone 8 cells, where the RT peak was reached 9 to 10 days after infection, the HUT-78, CEM-T4, and Jurkat cell lines showed maximal RT values 2 to 5 weeks after infection (Table 1). Southern blot and PCR analysis revealed the presence of relatively low amounts of MK6 provirus in the host cell genomes (data not shown). Furthermore, little or no cytopathic effects were observed in these three cell lines.

MK6 is highly infectious for macaque and human PBL

Eighteen days after infection of human PBL peak RT activity was present in the cell-free supernatants (Table 1). After cocultivation with uninfected Molt-4 clone 8 cells giant cell formation and high RT values of about 60 pmol dGMP incorporation per milliliter of supernatant were observed. The infection of macaque PBL with MK6 proceeds much faster. Seven days after infection of rhesus and fascicularis macaque PBL, RT activities

comparable to the peak activity observed in infected human PBL culture supernatants were detected in both cultures (Table 1). Since macaque PBL were very sensitive, subsequent cocultivation with Molt-4 clone 8 cells was necessary. About 10 days later the RT values increased to about 75 pmol dGMP incorporated per milliliter of supernatant. MK6 proviral DNA in these cultures was detected by PCR analysis (Fig. 3).

The phylogenetic position of HIV-2_{BEN}

To investigate the phylogenetic position of HIV-2_{BEN} relative to other HIV-2 and SIV isolates, evolutionary trees were constructed from the DNA sequences of the *env* (Fig. 4A) and *gag* (not shown) genes. The branching order is consistent for both genes and also for different methods of evaluation of the pair-distance matrix (i.e., using transversions only, as well as both weighted and unweighted transitions and transversions). The diagram indicates that HIV-2_{BEN} has branched off the main stem earlier than the HIV-2 isolates previously described. From the groups HIV-1, HIV-2, and SIV, the four sequences furthest diverged from their assumed common precursor were compared by using a method of noise analysis (Eigen *et al.*, 1988) (Fig. 4B). The diagram reveals a bundle-like topology since the three box dimensions are of almost equal magnitude. All four groups of HIV and SIV (HIV-1, HIV-2/SIV_{SMM/MAC}, SIV_{AGM}, and SIV_{MND}) branch off in-

FIG. 4. Comparative sequence analysis of HIV-1, HIV-2, and SIV based on the *env* DNA sequences in the purine/pyrimidine space (1992 base positions considered). (A) Phylogenetic tree derived by cluster analysis. In the scale the number of diverged base positions is given. Therefore, the distance in sequence positions between any two isolates is twice the distance along the ordinate to the connecting node. (B) Exact geometry of the four most diverged sequences. Numbers refer to exact positions as described by Eigen *et al.* (1988).

side the shaded area in Fig. 4B. We therefore conclude that there exists a common node for the four groups, which lies within the box. The classical tree construction methods usually yield a definite order of even the early branching nodes (Smith *et al.*, 1988; Tsujimoto *et al.*, 1989). However, the present analysis shows that due to noise accumulation, the existing data are not sufficient to specify the early branching order of HIV-1, HIV-2/SIV_{SMM/MAC}, SIV_{AGM}, and SIV_{MND}.

DISCUSSION

In the present study we describe the biological properties and the phylogenetic position of a new HIV-2 proviral clone.

The varying biological properties of HIV-2 isolates, such as replication rates, cytopathogenicity, and cell tropism may have relevance to the different degrees of pathogenicity in humans (Kong *et al.*, 1988; Kühnel *et*

al., 1989). Another HIV-2 isolate from a patient with neurological disorders, HIV-2_{D194} (Kühnel *et al.*, 1989), grows slowly with little or no syncytia formation in HUT-78 cells. Our data show that those findings could not be generalized for other human T-cell lines.

Inexpensive and efficient animal models are necessary for the development of protective vaccines against HIV infection. To date, only macaques infected with various SIV isolates have developed AIDS-like symptoms (Daniel *et al.*, 1987; McClure *et al.*, 1989), while the pathogenicity of HIV-2 for macaques is still a subject of discussion (Letvin *et al.*, 1987; Dormont *et al.*, 1989). Nevertheless, clinical alterations in HIV-2-infected macaques have been described (Dormont *et al.*, 1989) and the duration of the animal studies conducted so far may be too short to exclude a subsequent appearance of AIDS-like symptoms. Therefore, the development of an animal model with a well-characterized molecular clone of a human virus, highly related to SIV_{SMM/MAC}, will be of great interest. Of the molecular HIV-2 clones described previously (Naidu *et al.*, 1988; Zagury *et al.*, 1988), only the HIV-2_{SBL/ISY} clone (Franchini *et al.*, 1989) has been reported to be infectious for macaque PBL and macaques, though the data have yet to be published.

We have shown that MK6 reproducibly replicates in the PBL of rhesus and cynomolgus macaques. Preliminary data has proven that MK6 is also infectious for cynomolgus monkeys. Inoculation of cell-free MK6 virus in two animals led to seroconversion 4 weeks post-inoculation and viremia in one animal (data not shown).

The MK6 clone has several interesting biological features and provides us with many opportunities for future study. It is highly cytopathic and blocks proliferation in Molt-4 clone 8 and MT-2 cells. Therefore, antiviral agents and neutralizing sera can be tested *in vitro*. Since MK6 is infectious not only in human and macaque PBL, but also for macaques, we will investigate the influence of the negative regulatory *nef* protein (Guy *et al.*, 1987) on the *in vitro* and *in vivo* pathogenicity of HIV-2. To examine the genetic evolution of the HIV-2 genome, we have begun to determine the *in vivo* and *in vitro* mutation rates of MK6 by PCR analysis.

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Note added in proof. After submission of our manuscript to *Virology* a further HIV-2, called HIV-2_{ALT}, which has branched off the main stem of HIV-2 earlier than HIV-2_{BEN} has been published. (Dietrich U. *et al.*, 1989, *Nature* 342: 948–950.)

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