# Characterisation of Primary Isolates and Infectious Molecular Clones of Human Immunodeficiency Virus Type I from Cameroon

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Professor Dr. U. Wienand
Dekan

To my parents,
for giving me a meaningful education

# 1. INTRODUCTION

## 1.1 The human immunodeficiency virus as member of the Retroviridae family

The human immunodeficiency virus (HIV) is the etiological agent of the acquired immune deficiency syndrome (AIDS). The first cases of this then new disease condition were reported 20 years ago (Gottlieb *et al.*, 1981) and the isolation of the virus followed shortly afterwards (Barré-Sinoussi *et al*., 1983). By the end of the year 2000, over 36 million people had been infected with this virus globally, with about 70 % of these living in sub-Saharan Africa. Over 3 million deaths were reported as a result of AIDS (UNAIDS 2000). Despite its side effects, combination antiretroviral therapy has afforded many individuals in the industrialised nations clinical relief. However, the contrary is observed in the developing world which portray the highest rates of HIV infection and yet lack access to these agents.

HIV belongs to the Lentivirus genus of the Retroviridae family. Retroviruses contain the reverse transcriptase enzyme that uses the viral genomic positive stranded RNA as a template for synthesis of linear double-stranded proviral DNA which is later integrated into the genome of the cell and serves as the basis for viral gene expression. Based on nucleotide sequence relationship and genome structure, retroviruses can be divided into seven genera (table 1.1).

Lentiviruses differ from other retroviruses by their unusual morphological characteristics (cylindrical or cone shaped capsid), two phases of gene expression and the possession of a complex genome with several accessory and regulatory genes (Coffin *et al.*, 1997). HIV infection is characterised by insidious disease induction, persistence through active replication, latency, recombination and variation. This variation is manifested by the presence of immune escape variants, drug resistance variants and variants which use different cell surface receptors in addition to CD4. All these characteristics are reflected in the sequence variability of HIV which is an ongoing evolutionary process.

## 1.2 HIV genomic organisation

The HIV-1 genome encodes nine open reading frames (fig. 1.1). Three of these encode the Gag, Pol and Env polyproteins common to all retroviruses, which are

| Genus | Example | Virion morphology | Genome |
|---|---|---|---|
| Avian sarcoma and leucosis group | Rous sarcoma virus (RSV) | Central, spherical core “C particles” | simple |
| Mammalian B-type viral group | Mouse mammary tumor virus | Eccentric spherical core “B particles” | simple |
| Murine leukemia-related group | Moloney murine leukemia virus | Central spherical core “C particle” | simple |
| Human T-cell leukemia-bovine Leukemia group | Human T-cell leukemia virus | Central, spherical core | complex |
| D-type group | Mason-Pfizer monkey virus | Cylindrical core “D particles” | simple |
| Lentiviruses | Human and simian immunodeficiency viruses | Cone-shaped core | complex |
| Spumaviruses | Human foamy virus | Central, spherical core | complex |

**Table 1.1**: Classification of retroviruses (Coffin *et al.*, 1997).

subsequently proteolyzed into individual proteins. The four Gag proteins, matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and the two Env proteins, surface or gp120 (SU) and transmembrane or gp41 (TM), are the structural components that make up the core of the virion and outer membrane envelope. The three Pol proteins, protease (PR), reverse transcriptase (RT), and integrase (IN) provide essential enzymatic functions and are also encapsulated within the particle (fig. 1.1). Like other so-called complex retroviruses HIV encodes several accessory proteins. The early gene product Tat activates viral transcription in that it functions as a transcriptional elongation factor. Rev mediates transport of incompletely or unspliced viral RNAs into the cytoplasm. Apart from several still disputed functions, Nef enhances viral infectivity through CD4 down-regulation *in vivo*. Vif enhances viral infectivity, Vpr mediates the rapid transportation of nucleoprotein complexes into the host cell nucleus and Vpu promotes degradation of CD4 in the ER during env synthesis (Frankel and Young 1998). The proviral DNA of HIV is terminally redundant with two

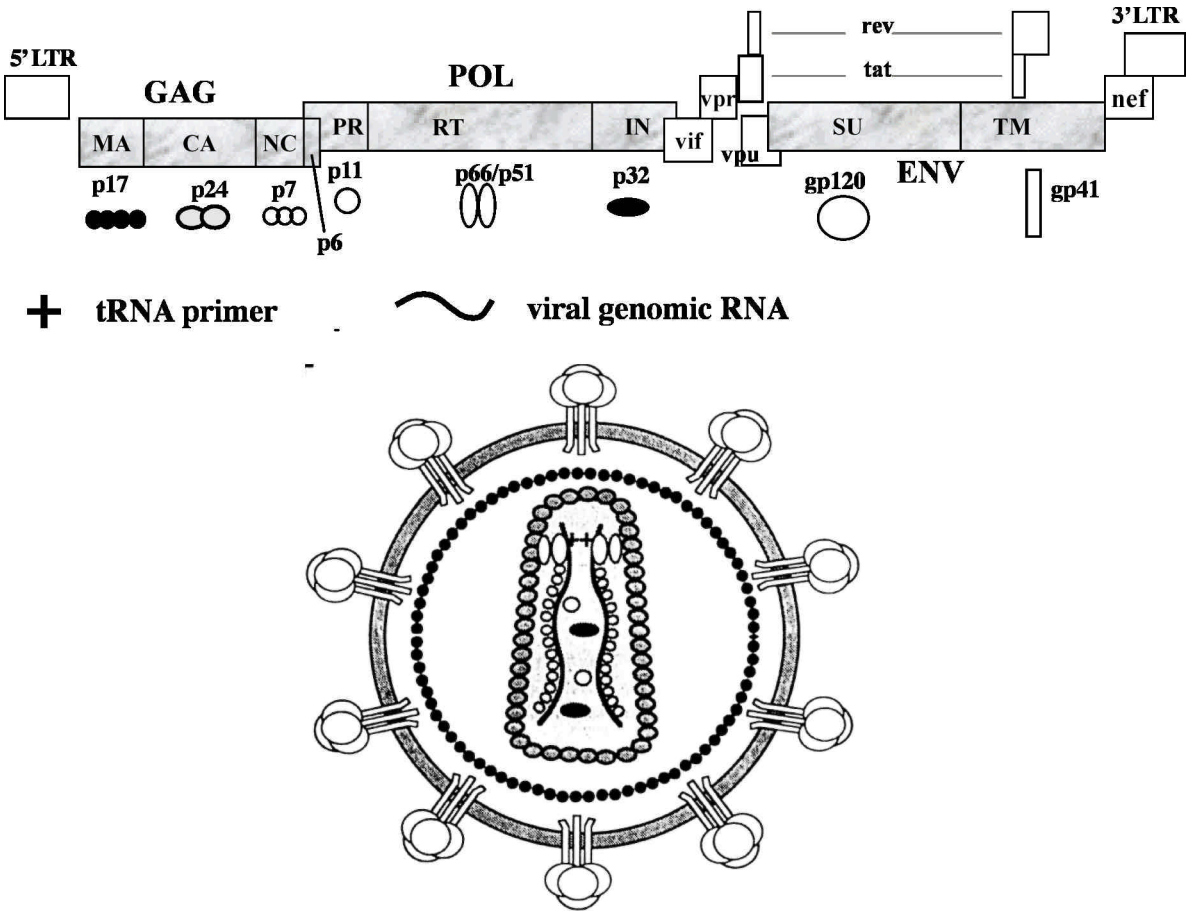


**Fig. 1.1:** Genomic organisation of HIV-1. Top: The open reading frames of the viral proteins are depicted. The virus structural genes are shaded and their respective products after cleavage are indicated below them. The accesssory genes and the LTRs are shown as open boxes. MA: matrix; CA: capsid; NC: nucleocapsid,;PR: protease; RT: reverse transcriptase; IN: integrase; TM: transmembrane glycoprotein; SU: surface glycoprotein. Below is a typical representation of a mature HIV particle: spherical, about 100 nm in diameter, and consists of a lipid bilayer membrane surrounding a conical nucleocapsid. Adapted from Coffin *et al*., 1997.

identical long terminal repeats (LTRs) which contain the promoter sequences and numerous regulatory elements. They comprise U3, R, and U5 (unique 3', repeat, unique 5') sequences. The proviral DNA genome of HIV is 9.7 kb long (fig. 1.1).

### 1.3 HIV life cycle

The life cycle of HIV commences with the attachment of the envelope glycoprotein gp120 to the target cell receptors. The CD4 molecule and one of several chemokine receptors (Klatzman *et al*., 1984; Feng *et al*., 1996) mediate attachment of the virion. Following binding, the transmembrane gp41 undergoes a conformational change that promotes virus-cell membrane fusion thereby allowing entry of the core into the cell. The virion core is uncoated to expose a viral nucleoprotein complex probably

containing MA, RT, IN, Vpr, and RNA. Shortly after uncoating reverse transcription begins whereby the genomic RNA is converted by viral reverse transcriptase into a duplex linear DNA. In the nucleus, IN then catalyses integration of the viral DNA into a host chromosome. Transcription is achieved by cellular RNA polymerase II producing viral RNA transcripts which are expressed from the promoter located in the 5'LTR with Tat greatly enhancing the rate of transcripts produced. Through Rev regulation a set of spliced and genomic-length RNAs are transported from the nucleus to the cytoplasm where they are translated. The env mRNA is translated at the endoplasmic reticulum. The Env products are later cleaved at the Golgi apparatus by cellular proteases and become localized to the cell membrane where they are assembled together with the Gag and Gag-Pol polyproteins, Vif, Vpr, Nef, and genomic RNA. Budding of the immature particles at the cell membrane is followed by a morphological change termed maturation of the virion which involves proteolytic processing by viral PR of the Gag and Gag-Pol polyproteins into MA, CA, NC, p6, PR, RT, IN. The mature virion is then ready to infect another cell (Coffin *et al*., 1997; Frankel and Young, 1998).

### 1.4 Overview of reverse transcription in HIV

Reverse transcription starts shortly after the uncoated virion releases its content into the cytoplasm (Luciw, 1996; Coffin *et al*., 1997). A cellular primer $tRNA^{Lys}$ is annealed to the primer binding site (PBS) which is located at the 5' end of the template ssRNA (fig. 1.2, step A). The viral RT first copies U5 and R sequences forming minus-strand strong-stop DNA. A ribonuclease (RNAse H) activity in reverse transcriptase degrades the U5 and R of the template RNA exposing minus-strand strong-stop DNA which then transfers to the 3' end of the RNA genome, presumably using complementarity between the R regions (step B). Minus-strand DNA synthesis continues (step C) and the RNAse H then degrades the RNA template except for two polypurine tracts (PPT); one is located at the 3' end of *pol* (central PPT) and the second near the U3 region of the 3' LTR (step D) (Charneau *et al.*, 1992). These two PPT act as primers initiating plus-strand DNA synthesis which starts from the PPT and proceeds to the 5' end of the template forming the plus-strand stop DNA. In this process, the minus strand U3-R-U5 DNA as well as the portion of the primer tRNA that is complementary to the PBS are copied. This plus-strand stop DNA transfers to

the 3' end of the almost completed minus-strand DNA presumably by complementarity to the PBS region (step E). The synthesis from the cPPT continues

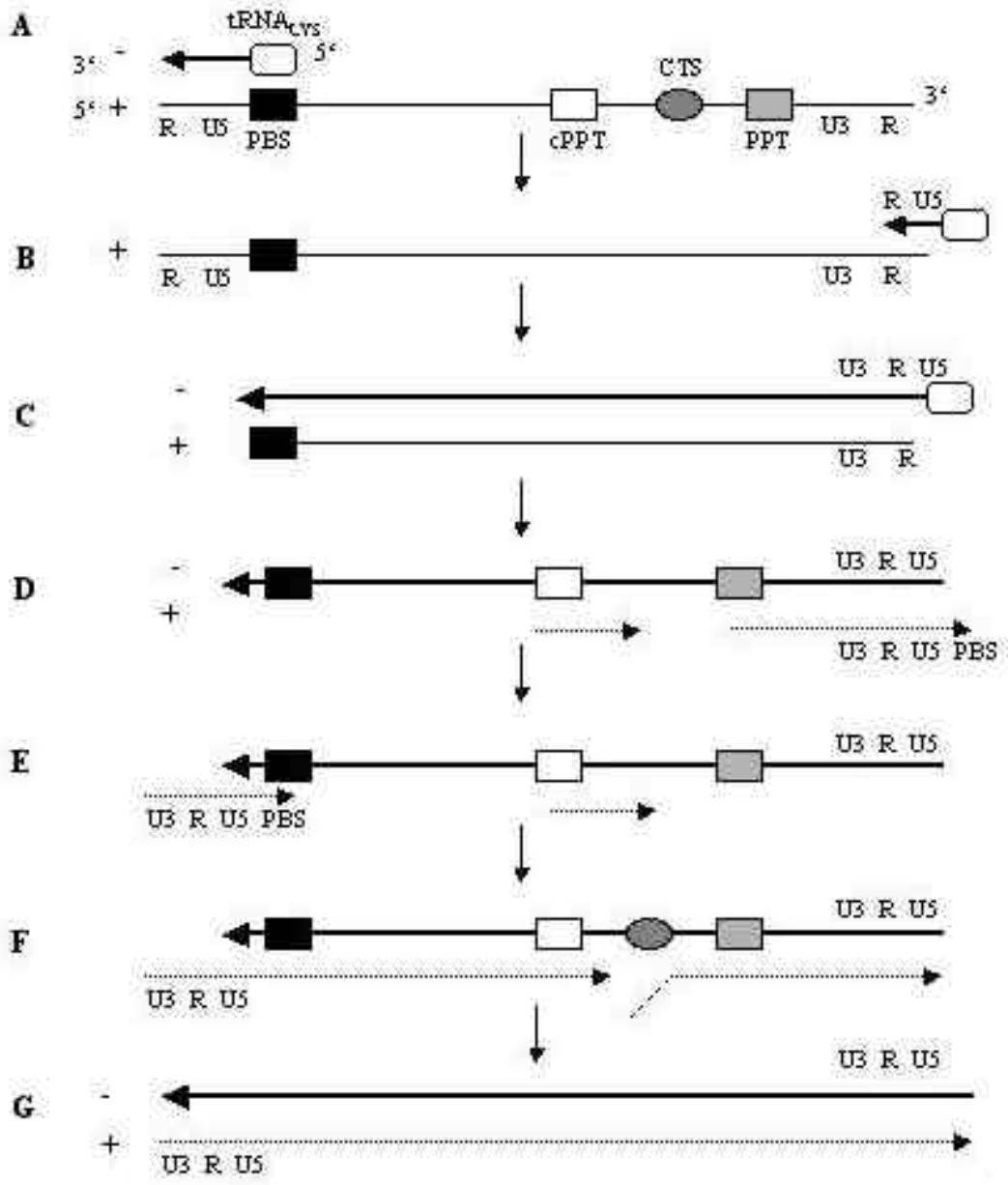


**Fig. 1.2:** Generation of a dsDNA copy (provirus) from a ssRNA template by reverse transcription. Thin line = RNA, thick line = minus strand DNA, dotted line = plus strand DNA, cPPT = central polypurine tract, PBS = primer binding site, CTS = central termination signal. Adapted from Luciw, 1996.

to the 5' end of the minus template while the synthesis from the plus-strand DNA continues to the central termination signal (CTS) located at the 3' end of *pol* after displacing in its course a short stretch of synthesised plus strand DNA that was started from the cPPT RNA primer (step F). DNA synthesis of both plus and minus strands then proceeds to form a complete copy of the retroviral RNA with two LTRs

(step G). This provirus is then ready for integration mediated by the integrase (Goff, 1992; Coffin *et al*., 1997).

### 1.5 HIV target cells

T-cells are divided into two subsets. The T-helper/inducer cells which carry the CD4 marker and the cytotoxic T-cells identified by their CD8 marker (Roitt *et al*., 1998). HIV infects cell types expressing CD4, like T-helper lymphocytes, macrophages, monocytes, dendretic cells and other phagocytic cells. The CD4 receptor is the primary receptor of HIV (Klatzman *et al*., 1984). Productive HIV infection requires specific activation of the T-cells. Uninfected healthy individuals carry about 1,200 CD4+ T-lymphocytes / µl of blood. However, at all times during HIV-infection and AIDS disease progression, this number steadily decreases due to depletion of these cells to levels <200 CD4+ cells / µl in AIDS patients with occasionally no cells detectable when the final stages are reached (Gottlieb *et al*., 1981; Castro *et al*., 1992). The number of CD4+ cells in infected individuals is therefore a prognostic marker for disease progression during AIDS. CD4 is necessary and sufficient for attachment to cells, however, its presence alone is clearly not sufficient for viral entry, since not all human cells engineered to express CD4 are susceptible to HIV infection (Chesebro *et al*., 1990; Clapham *et al*., 1991). It was therefore evident that additional cell surface components besides CD4 were essential for fusion. The description of CCR5 and CXCR4 the two main co-receptors respectively needed for HIV entry have provided new insights into the understanding of HIV infection (Feng *et al.*, 1996).

### 1.6 HIV-1 envelope glycoproteins and entry events

The binding of CD4 and gp120 induces conformational changes in the latter that contribute to the exposure of a binding site for either of several chemokine receptors. The main co-receptors are CCR5 and CXCR4, but there are at least nine others namely CCR2b, CCR3, CCR8, GPR1, BOB (GPR15), BONZO (STRL33), US28, V28 and ChemR23 which have been shown to support entry of one or more virus strains. The interaction of gp120 with co-receptor triggers Env to undergo another conformational change leading to extension of gp41 and insertion of its fusion peptide into the target cell membrane. The gp120 contains five variable loops designated V1-V5 interspersed with five relatively conserved regions designated

C1-C5 (Starcich *et al.*, 1986; Willey *et al.*, 1986). Studies of chimeric envelope glycoproteins demonstrated that the third variable (V3) loop of gp120 is a major determinant of tropism (Feng *et al.*, 1996). It becomes exposed upon CD4 binding and presumably interacts with the cognate receptor (Clapham, 1997). Studies have shown that V3 deleted versions of gp120 do not bind CCR5, even though CD4 binding occurs at wild-type levels. V3 however functions in concert with other gp120 regions including V1, V2, and C4 (Labrosse *et al.*, 2001).

### 1.7 HIV-1 chemokine receptors, viral tropism and genotype

Most primary clinical isolates of primate immunodeficiency viruses use the chemokine receptor CCR5 for entry (Feng *et al.* 1996). For most HIV-1 isolates that are transmitted and that predominate during the early years of infection, CCR5 is an obligate co-receptor. Approximately 1 % of Caucasians are genetically deficient in CCR5 expression in that a homozygous 32 base pair deletion provides a relative resistance to HIV-1 infection (Bjorndal *et al*., 1997). CCR5-tropic isolates are also known as M-tropic or non-syncytium inducing (NSI) because they can infect both macrophages and primary CD4+ T-cells, but not transformed T-cell lines and are unable to form syncytia on MT-2 cells or in certain commonly used T-cell lines (Schuitemarker 1991; fig. 1.3). HIV-1 isolates arising later in the course of disease, on the average 4-5 years post infection, often use other chemokine receptors. CXCR4 is the most frequently used, in about 50 % of HIV infected individuals, and can infect primary T-cells and T-cell lines forming syncytia in MT-2 cells (SI). While this shift in tropism can sometimes be accompanied by a loss of ability to infect macrophages, more often primary isolates retain this property and are able to use both CCR5 and CXCR4 for entry into target cells and are therefore referred to as dual-tropic (fig. 1.3). The emergence of these virus types correlates with accelerated disease progression (Connor, 1993). Viruses that are well adapted to grow on transformed cell lines by continuous passage are referred to as T-cell Line adapted (Berger *et al*., 1999).

The use of CCR5 by a diverse group of immunodeficiency viruses, with divergent V3 sequences first suggested the involvement of more conserved gp120 sequences. Apart from the V3 region, studies have also implicated other variable domains, of gp120 as determinants of chemokine receptor usage (Kwong *et al.*, 1998; Labrosse *et al.*, 2001).

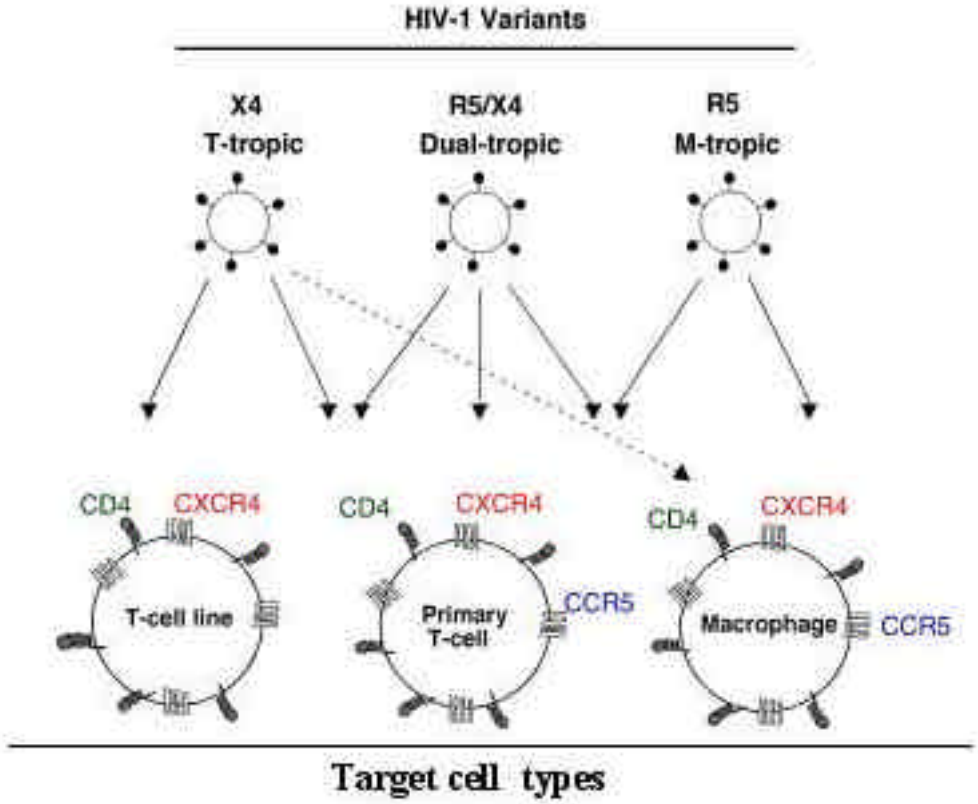


**Fig.1.3:** Model for co-receptor usage and tropism. T-tropic strains are specific for CXCR4 and can infect continuous CD4+ T cell lines and primary CD4+ T cells. M-tropic strains are specific for CCR5 and can infect primary macrophages and primary CD4+ T cells. Dual tropic strains can use both CXCR4 and CCR5, and can infect continuous CD4+ T cell lines, macrophages and primary T cells (Berger *et al*., 1997).

Virtually all primary HIV-1 isolates use either CXCR4 or CCR5, or both co-receptors (Zhang *et al.*, 1996, Connor *et al.*, 1997, Bjorndal *et al.*, 1997) for entry. Isolates from different geographical regions display little relationship between genetic subtype and co-receptor usage (Zhang *et al.*, 1996, Bjorndal *et al.*, 1997, Bazan *et al.*, 1998) although some distinctions have been reported (Tscherning *et al.*, 1998). More often, a marked correlation is observed between biological phenotype and co-receptor usage. Besides these main co-receptor, HIV-2 and SIV usually use in addition, one or more of the other known chemokine receptors**.** CD4 independent HIV-2 isolates which utilise either CXCR4 or CCR5 for entry have been described (Endres *et al.*, 1996; Reeves *et al.*, 1997; Bandres *et al.*, 1998). Recently, a primary HIV-1 isolate that required neither CD4 nor any known chemokine receptor but CD8 for entry was reported (Saha *et al.*, 2001).

### 1.8 HIV drug targets

Lentiviruses provide a number of possible targets for pharmacological intervention such as virion-cell receptor interaction, reverse transcription, integration, proteolytic maturation and virion-specific transcriptional sequelae. Nowadays, HIV replication can be drastically reduced through the administration of potent combination of antiretroviral drugs that target reverse transcriptase and protease (Raboud *et al*., 1998). Sustained inhibition of viral replication results in partial reconstitution of the immune system in most patients, substantially reducing the risk of clinical disease progression and death. Reservoirs of HIV in latently infected resting T-lymphocytes and other long-lived cell populations however make it unlikely that HIV can be eradicated from the body by antiretroviral therapy alone (Finzi *et al*., 1997). This not withstanding, the availability of Highly Active Antiretroviral Therapy (HAART) since 1996 has brought a dramatic change to the natural history of HIV with an unprecedented drop in disease progression and mortality in Western countries (Palella et al., 1998; Pezzotti et al., 1999). HAART involves the administration of a combination of non-nucleoside or nucleoside RT analogues and/or protease inhibitors. In developing countries, the benefits of antiretroviral therapy are still far from being achieved due to the cost and affordability of these medications. These countries also lack the necessary infrastructure to monitor for the development of drug resistance in patients being treated (van der Groen, 2001)

Individuals carrying polymorhisms in the targeted genes as a result of sub-optimal drug therapy may show reduced sensitivity to therapy. Several studies investigating the frequency of drug related polymorhisms in HIV-1 infected drug-naive individuals or isolates from individuals recently infected with HIV-1 subtype B strains have found several drug resistant-associated mutations depending on the regimen used (Condra *et al.*, 1995; Birk and Sonnerborg, 1998). Reports indicate that up to 26% of some drug-naive individuals harbour population with modest 3-10 fold reduction in susceptibility to at least one drug, implying that drug susceptibility testing prior to treatment of drug-naive individuals may become necessary (Yerly *et al.*, 1999). However, the prevalence of drug resistant polymorphisms among non-subtype B strains of HIV-1 is currently largely unknown.

Another potential therapeutic strategy to combat HIV infection being investigated is the use of HIV entry inhibitors. Early *in vitro* experiments using monoclonal antibodies directed against CD4 as well as a soluble version of CD4 (sCD4) blocked HIV

infection indicating the dependence of HIV on the CD4 protein for infection and its potential role as a therapeutic target (Dalgleish *et al.*, 1984). However follow-up experiments using primary, non-laboratory adapted isolates of HIV-1 showed poor neutralisation by sCD4 (Daar *et al.*, 1990). The ß-chemokines, RANTES, MIP-1α and MIP-1β produced by CD8+ T-cells are ligands of CCR5 and therefore potent inhibitors of infection by CCR5 HIV-1 strains (Cocchi *et al.*, 1995, Alkahtib *et al*., 1996). SDF-1 on the other hand is the ligand of CXCR4 and inhibits infection by respective viruses (Feng *et al.*, 1996). Ligands to co-receptors including chemokines, monoclonal antibodies and small organic molecules are able to block HIV entry and fusion, although at times inhibition depends on virus strain and cell type (Donzella *et al.*, 1998; McKnight *et al.*, 1997).

### 1.9 Classification of HIV and other primate lentiviruses

On the basis of their genomic organisation and phylogenetic relationship with other primate lentiviruses, human immunodeficiency viruses have been divided into two types, HIV-1 and -2 (fig. 1.4). Current classification based on full-length genome analysis sub-divides HIV-1 into three different groups; designated M (major), O (outlier) and N (non-M / non-O), with the most predominant group M further classified into subtypes A-D, F-H, J-K and F further subdivided into F1 and F2 (Robertson *et al.*, 2000; Gürtler *et al.*, 1994; Simon *et al.*, 1998). Comparison of the protein coding sequences of HIV-1 subtypes indicate that they differ from each other on average by 15, 10 and 22 % in Gag, Pol and Env respectively (Gao *et al.*, 1997). Between the sub-subtypes the differences in Gag and Env are 10% and 15% respectively (Robertson *et al.*, 2000). In addition there are several groups of “Circulating Recombinant Forms” (CRFs) which are viruses resulting from recombination events between different subtypes. The term CRF defines groups of viruses that are widely distributed and share a common recombinant lineage (Robertson *et al.*, 2000). To date there are 11 different forms of CRFs with the mosaic structure of some composed of more than two genetic subtypes. These have been termed “complex recombinants” (CRFcpx; Peeters, 2000). Group O viruses show much variability between isolates and have not been classified into subtypes as it is the case with group M viruses. HIV-1-N is the most recent group to be described and is represented by two strains from Cameroon (Simon *et al.*, 1998) which were shown to be composed of a mosaic between HIV-1-M and SIVcpz (Gao *et al.*, 1999).

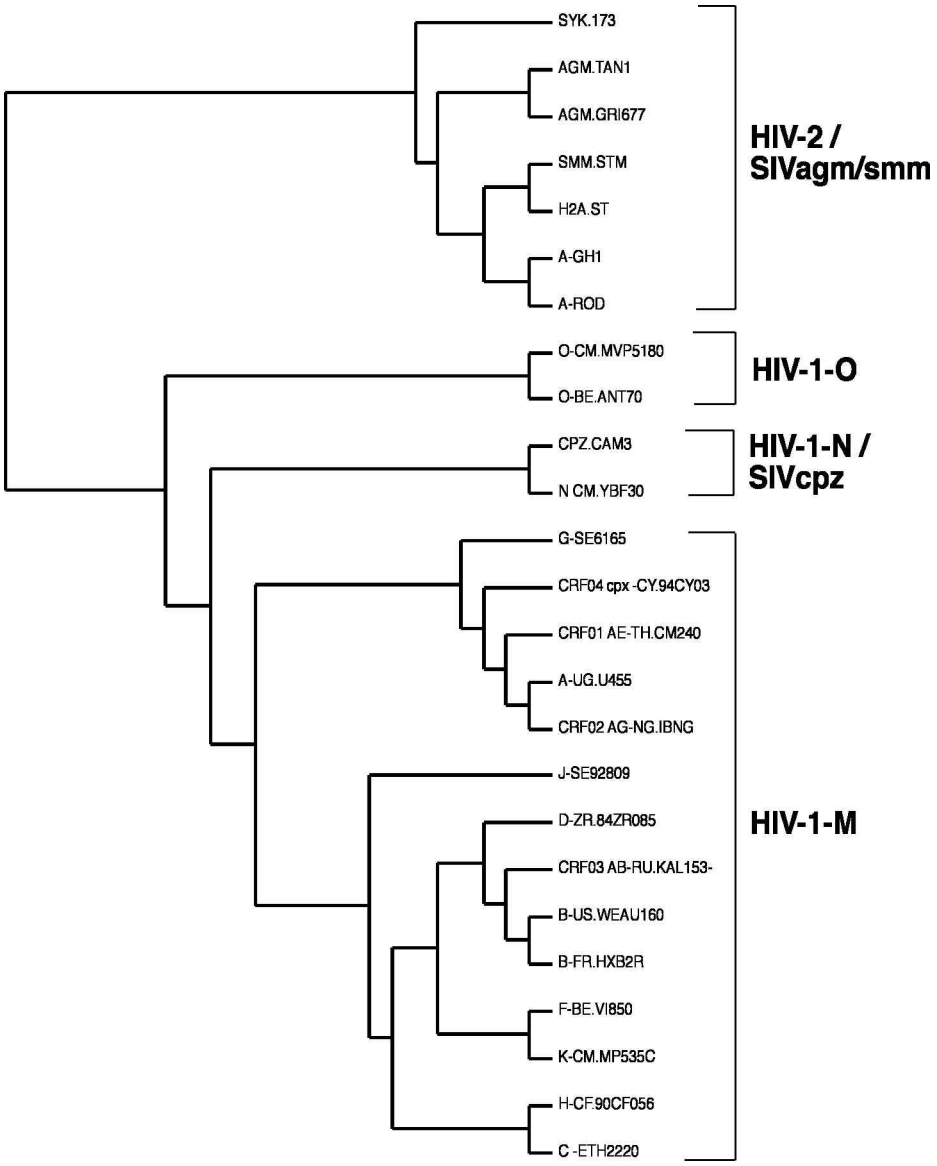


**Fig.1.4:** Unrooted neighbour-joining phylogenetic tree of full-length *pol* sequences showing the major lineages of primate immunodeficiency viruses. The three groups of HIV-1 (M, N, O) as well as HIV-2 and SIVagm/smm are indicated to the right. Within HIV-1 group M, the various subtypes and CRFs are shown at the beginning of each isolate.

HIV-2 on the other hand is sub-classified into subtypes A through F. Genetically distinct lentiviruses termed simian immunodeficiency virus (SIV) have been isolated from non-human primates species including African green monkeys (*Cercopithecus aethiops spp.*; SIVagm), sooty mangabeys (*Cercocebus atys*; SIVsm), mandrills (SIVman; *Papio sphinx*), and chimpanzees (*Pan troglodytes troglodytes*; SIVcpz).

It is believed that the various lineages of HIV were introduced into the human population through different independent zoonotic (cross species) transmission events (Gao et al., 1999). HIV-2 is more closely related to SIVsm from sooty mangabeys than it is to HIV-1 while HIV-1 group M is closely related to the SIVcpz

sequences from chimpanzees (Hahn *et al*., 2000). However, the degree of sequence homology between HIV-1 group M and SIVcpz is lower than between HIV-2 and SIVsm (Gao *et al.*, 1999). On average, HIV-1 and –2 share only 40-60 % sequence homology in the major open reading frames (ORFs).

### 1.10 Mechanisms of HIV genetic variability

Retroviral populations generally evolve rapidly in order to fit the changing requirements of the environments in which they replicate, thereby altering their tropism, evading the defences of the host or adapting to survive drugs designed to block their replication (Wain-Hobson, 1993). HIV exhibits tremendous genetic variability in its host, due mainly to mutations caused by the error-prone nature of the virus replication machinery and recombination between pre-existing viral populations. The continuous and rapid HIV turnover which averages $10^9$ copies per day, leads to the production of a complex mixture of highly related yet genetically distinct viruses termed “quasispecies” present in an individual (Perelson *et al.*, 1996; Wain-Hobson, 1992).

#### 1.10.1 Mutations

Mutations (point mutations, deletions, insertion, duplications, frameshifts) occur mostly during the process of reverse transcription and are due to the low fidelity of the viral reverse transcriptase which lacks 3’- 5’ exonuclease proof reading activity and is thus more error prone than cellular DNA polymerases (Preston *et al.*, 1988). The *env* gene of HIV allows most variations where nucleotide and amino acid substitution accumulate at an average rate of about 1 and 2.5 % per year respectively (Burns and Desrosiers, 1991). Mutations in *env* tend to be localised to discrete variable domains within which about 95% of the nucleotide substitution change an amino acid. The majority of mutations observed in HIV are substitutions, with G to A transitions by far the most common. This is the probable reason for the high “A” content of retroviral genomes. Frameshift mutations occur less frequently than substitution mutations and involve the addition or deletion of a nucleotide in a homopolymeric run (Pathak and Temin, 1990a,b). About one in 10 genomes suffers a significant internal deletion during a single round of replication (Olsten *et al.*, 1990;

Parthasarathi *et al.*, 1995). Deletions are strongly potentiated by the presence of direct repeats (Delviks and Pathak, 1999).

### 1.10.2 Recombination

HIV-1 like all other retrovirus particles contain two RNA strands of positive polarity which typically are derived from the same parental provirus. Infected cells can simultaneously harbour two different proviruses therefore allowing one RNA transcript from each provirus to be encapsidated into a single heterozygous virion. Templates switching by RT during reverse transcription can give rise to recombinant progeny within such heterozygous virions (Hu and Temin; 1990). This phenomenon can lead to recombination within HIV-1 subtypes (intra-subtype recombination; Morris *et al*., 1999) between subtypes (inter-subtype recombination; Peeters 2000) or between groups (inter-group recombination; Takehisa *et al*., 1999). Inter-subtype or inter-group recombination are most common in geographical regions where multiple types of HIV exist in dually infected individuals. Despite reports of HIV-1 and HIV-2 dually infected individuals, no recombinants between these two types have been reported. It is estimated that as many as 10 % of all HIV-1 isolates may be inter-subtype recombinants (Robertson *et al.*, 1995).

High rate of recombination is probably the most remarkable feature of retroviral genetics. Recombination occurs following infection with heterogeneous virions, i.e. those produced by cells co-infected with two parental isolates. There are presently two different models that have been proposed for the mechanism of retroviral recombination: the forced copy-choice and the strand displacement assimilation model. The “forced copy choice” model proposes that when the genomic RNA in the virion is damaged, the RT encounters a break in the viral RNA and therefore switches to the other copy of the genomic RNA thereby salvaging the encoded genetic information. This occurs during minus-strand DNA synthesis (Coffin, 1979; Hu and Temin 1990). The “strand displacement assimilation” model proposes that DNA fragments are displaced during plus-strand DNA synthesis from one template and subsequently assimilated by the plus strand DNA synthesis from the other template causing recombination (Junghans *et al*., 1982). The process of recombination may therefore increase the variation in a population by combining different variants and repairing damaged genes (Stahl, 1987).

### 1.11 Global distribution of HIV variants

Genetic variability studies indicate that Central Africa has the most heterogeneous forms of HIV (fig. 1.5A). In Cameroon, Gabon, Congo, Equatorial Guinea, Democratic Republic of Congo and the Central African Republic, all the different groups of HIV-1 uncovered so far can be found (Nkengasong *et al*., 1994; Murphy *et al*., 1993; Heyndrickx *et al*., 2000; Mboudjeka *et al*., 1999a; Vidal *et al*., 2000; Janssens *et al.*, 1997). SIV chimpanzee sequences show close similarity to HIV-1-M. The geographical habitat of this ape species is Central Africa, indicating that the first transmission from non-human primates to humans might have taken place in this region (Corbet *et al.*, 2000; Gao *et al.*, 1999). The group O viruses have their epicentre in Cameroon with only sporadic cases being reported in neighbouring countries (Peeters *et al.*, 1997), and the only two group N sequences obtained to date are from Cameroonians as well (Simon *et al.*, 1998). In West Africa the number of subtypes seems to be limited, dominated mostly by the CRF02.AG viruses and its parental subtypes A and G (Montavon *et al.*, 2000). HIV-2 is also present in this region, but less prevalent than HIV-1 probably due to its reduced pathogenicity and rate of perinatal transmission (Kanki, 1994). In Southern Africa, the most affected region in sub-Saharan Africa, subtype C is most prevalent. This subtype which is also quite common in Asia comprises about 48 % of HIV-1 subtypes globally. Several studies have proposed correlation of viral structural characteristics of subtypes and the mode of transmission as the probable reason for the widespread nature of certain subtypes. Follow-up studies have proven most of these reasons to be unlikely (Kunanusont *et al.*, 1995; Soto-Ramirez *et al.*, 1996; Dittmar *et al.*, 1997a). Most Asian countries are dominated by the CRF01.AE viruses while there is a new epidemic involving a B/C recombinant in China (Gao *et al.*, 1996; Piyasirisilp *et al.*, 2000; Su *et al.*, 2000). In Europe and America subtype B is the dominant strain, however cases of different subtypes or CRFs from individuals originating from regions harbouring multiple strains of HIV have been reported in Europe and America (Loussert-Ajaka *et al.*, 1995; Hampl *et al.*, 1995, Cornelissen *et al.*, 2000).

### 1.12 Cameroon and HIV: a brief background

Cameroon lies in West Central Africa along the Gulf of Guinea, between latitudes 2-12° north of the equator and covers a surface area of about 475,000 $km^2$ with a population of about 15 million people. As a result of its past, this country is a highly diversified entity of people, cultures, religions and languages. It comprises two English speaking and eight French speaking provinces. Moving from the south to the north of the country, the vegetation gradually changes from the dense equatorial forest through the savannah to the extreme arid northern zone around Lake Chad (fig. 1.5). Because of this extreme diversity, it has been described as “Africa in miniature”.

This description aptly fits the distribution of HIV subtypes in this country were HIV-1 studies showed the presence of almost all subtypes described so far in Africa (Nkengasong *et al*., 1994, Takehisa *et al*., 1998; Mboudjeka *et al*., 1999b). Additionally, the Central African chimpanzee species, *Pan troglodytes troglodytes* whose *env* sequences where recently described as closely related to HIV-1-N are also found in this country (Corbet *et al*., 2000; Gao *et al*., 1999). The western region of Cameroon has been described as a biogeographic transition zone, from *Pan troglodytes troglodytes,* the Central African chimpanzee species to *Pan troglodytes verus*, the West African chimpanzee species (Gonder *et al*., 1997). Briefly put, Cameroon is a “hotspot” for emerging lentiviruses.

About 7 % of the Cameroonian population is HIV infected and HIV-1 group M is the most dominant, while group O prevails in about 3-5 % of all HIV-infected cases (Zekeng *et al*., 1994; Nkengasong *et al*., 1994; Mauclere *et al*., 1997; Takehisa *et al*., 1998; Mboudjeka *et al*., 1999b, UNAIDS, 2000). The few cases of group N infections reported so far are from Cameroon (Simon *et al*., 1998). Dual infections with groups O and M which led to recombination have been described from this country (Takehisa *et al*., 1997; 1999; Peeters *et al*., 1999). Infections with HIV-2 are rare and are thought to be non-indigenous (Zekeng *et al*., 1992). Between 1986 when the first case of HIV was reported up till date, the prevalence of HIV and the number of reported AIDS cases has been on the rise (Zekeng *et al*., 1992; Mbopi-Kéou *et al*., 1998), although still lower than in other Eastern and Southern African countries like Uganda, Kenya, South Africa and Botswana were about a one-fifth of the population is HIV-1 infected (UNAIDS, 2000).

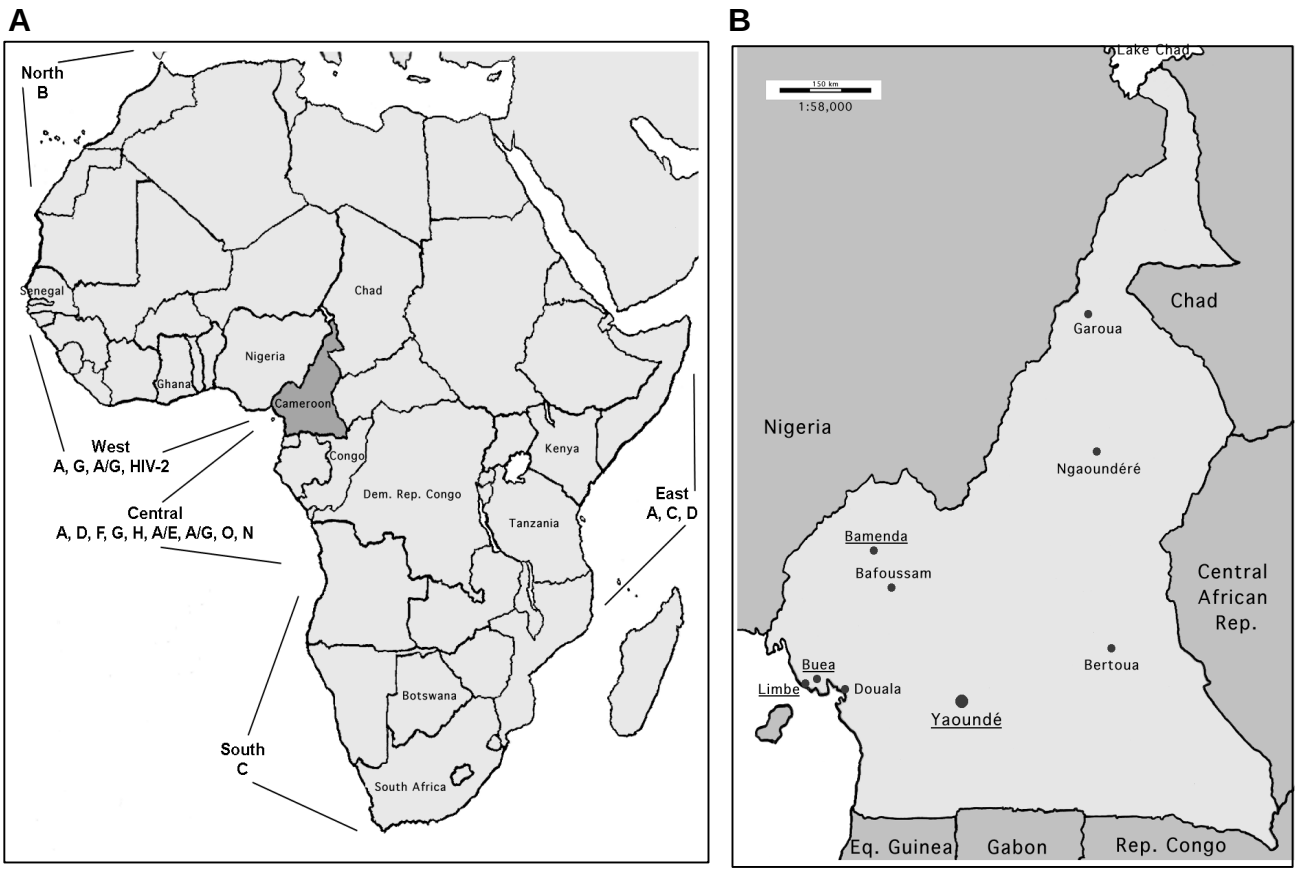


**Fig 1.5: A)** Location of Cameroon and the geographical distribution of HIV types, groups, subtypes and CRFs in the African continent. Subtype B is prevalent in the north; A, D and C in the east; A, G, CRF02.AG and HIV-2 in the west. Southern Africa has the highest HIV prevalence dominated by HIV-1 subtype C. However, the prevalence rate in Central Africa which harbours almost all HIV subtypes described to date is lower. Only the dominant forms are indicated. **B)** An enlarged view of Cameroon and its neighbouring countries. The regions from which the HIV-1 isolates originated are underlined.

The North West and South West Provinces are the only two English speaking regions of Cameroon. Based on their historical past and geographical location, these western provinces share close socio-economic ties with Nigeria, the anglophone western neighbour of Cameroon. They have also reported the highest number of AIDS cases between 1986 and 1995 (Mbopi-Kéou *et al*., 1998). However, until now, no details were available on the genetic subtypes that circulate in these two provinces. Delineating the subtypes is of prime importance in determining which genotype to be included in any future vaccine studies.

## 1.13 Aims of study

HIV displays important genetic variability. Differences between HIV-1 and HIV-2 in terms of transmissibility, pathogenesis and pattern of spread are fairly well documented (Kanki *et al.*, 1994). However, within and between groups of HIV-1 very little is known about the biological and functional differences that may be associated with this great sequence variability. These differences can be best studied using a panel of full-length infectious molecular clones whose properties are identical to the parental primary isolates which in turn reflect those of the virus found in an infected individual. Antiretrovirals curb the spread of HIV, however their successful use is hindered by the emergence of specific polymorhisms in PR or RT genes which affect drug susceptibility. Also since subtype specificity might be important for the development of efficient HIV vaccines, it is necessary to understand the geographical distribution of HIV subtypes especially from countries like Cameroon where “new” variants keep on emerging. Previous studies characterising HIV strains from Cameroon concentrated on the two major urban centres around Yaounde and Douala. This study was carried out in Western Cameroon, a region which lies between Central Cameroon where virtually all HIV groups and subtypes exist and Nigeria which portrays a limited number of HIV subtypes.

This study was aimed at:

-Collecting blood samples from HIV-positive drug-naive Western Cameroonians for subsequent analysis.

-Comparing the reactivity pattern of Western Cameroonian HIV-1 positive sera in a Western blot assay.

-Recovering primary HIV isolates from these blood samples by co-cultivation with human peripheral blood mononuclear cells thereby avoiding excessive passaging.

-Determining biological phenotypes including co-receptor usage of the primary isolates.

-Performing detailed genotypic analyses enabling phylogenetic classification of the isolates.

-Screening for naturally occurring drug resistance mutations in the protease and reverse transcriptase genes.

-Investigating the pattern of HIV-1 ”Circulating Recombinant Forms” (CRFs) found in this region by analysis of full-genome sequences thereby unveiling the crossover sites and possible recombination “hotspots”.

-Generating reference reagents particularly full-length infectious molecular clones of the dominant as well as peculiar forms of HIV-1 in Cameroon. Such clones will be important to evaluate the properties of and differences between HIV-1 groups and subtypes as well as for vaccine development.

# 2. MATERIALS AND METHODS

## 2.1 General

The chemicals used were from the following firms: Merck (Damstadt), Sigma (Deisenhofen), Serva (Heidelberg), Roth (Karlsruhe), Riedel de Haen (Seelze) and Roche (Mannheim).
Vacutainer tubes and needles were from Becton-Dickinson (Heidelberg), blood bags from NPBI-Emmer-Compascuum (The Netherlands), plastic wares from Greiner (Nürtingen), Becton-Dickinson (Heidelberg), Eppendorf (Hamburg), NUNC (Denmark).
Buffers were prepared using filtered water and at room temperature.
All HIV samples used in this study were obtained from HIV infected Cameroonians.

## 2.2 Biological analysis

### 2.2.1 Handling of infectious material

Virus cultivation and potentially infectious DNA material were handled in a level 3 laboratory while all other assays involving non-infectious materials were done in a level 2 laboratory according to stipulated guidelines for working in such laboratories.

### 2.2.2 Media and reagents for cell culture

Powder medium, trypsin, glutamine and antibiotics were purchased from Life Technologies (Eggenstein), fetal calf serum (FCS) PAA (Linz, Austria), dimethylsulfoxide (DMSO) Serva (Heidelberg), Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), interleukin 2 (IL-2) (Roche, Mannheim), phytohaemagglutinin (PHA; Sigma, Deisenhofen)

The following media and supplements were used for cell cultivation:
DMEM: Dulbecco’s Modification of Eagle’s Minimum Essential Medium
RPMI 1640: Roswell Park Memorial Institute 1640 Medium

Before use, the artificial media were supplemented with the following: 10 or 20 % fetal calf serum (FCS) which was inactivated for 30 mins at 56°C, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine.
Freezing medium for long term storage of cells: 50 % FCS, 40 % DMEM or RPMI 1640 and 10 % DMSO.
PBS (phosphate buffered saline): NaCl 9 g/l, $KH_2PO_4$ 0,21 g/l, $Na_2HPO_4$-$7H_2O$ 0.72 g/l, pH 7.3
Trypsin / EDTA solution: trypsin 0.5 g/l, EDTA 0.2 g/l PBS
PBS / EDTA: PBS with 0.5 mM EDTA sterilised by autoclaving.

Solutions for calcium phosphate transfection:
$CaCl_2$-solution: 250 mM $CaCl_2$ (sterilised by filtration).
BBS-solution: 50 mM BES, 280 mM NaCl, 1.5 mM $Na_2HPO_4$ (pH 6.95)

Standardised commercial ELISA kit:
HIV 1 + 2 Enzyme linked immunosorbent assay (ELISA; Dade-Behring, Marburg)
Solutions and reagents for “in-house” p24 ELISA assay:
Primary antibody: Ascites 183 (0.5 µg/ml)
Capsid standard (10 µg/ml) diluted serially (i.e. from 10 µg to 0.031 µg)
Secondary antibody: rabbit-anti-capsid (r α CA) + 100 µl PBS / Tween20, (10 % Tween20 + 10 ml PBS) + 10 ml newborn calf serum (NCS)
Tertiary antibody: anti-rabbit-peroxidase (α-r-PO) + 100 µl PBS / Tween 20 (10 % Tween 20 in PBS) + 10 ml NCS
Wash buffer: PBS / Tween 20 (10 % Tween 20 in PBS)
Blocking Buffer: 10 % NCS in PBS
Substrate: 2 µl $H_2O_2$ +100 µl TMB (10 mg 3,3`,5,5`-Tetramethylbenzidin / 1 ml DMSO) + 10 ml 0.1 M NaAc
Stop solution: 0.5 M $H_2SO_4$

Solutions and reagents for determination of co-receptor usage
Ice cold methanol / acetone (1:1)
Primary antibody: Ascite 183
Secondary antibody: goat anti-mouse IgG ß-galactosidase (ß-GAL) conjugate
Wash buffer: 1 % FCS / PBS

X-Gal: stock solution prepared by dissolving X-gal in dimethylformamide to make a 20 mg/ml solution.

### 2.2.3 Cell types and characteristics

Cell types used for different transfection and infection experiments

| Cell-type | Origin/source | Peculiarity / characteristic |
|---|---|---|
| HeLa P4 | Human carcinoma cell | Stably expresses CD4, CCR5 and CXCR4. Adherent, forms syncytia for SI isolates |
| 293T | Human carcinoma cell | Adherent, highly transfectable, lacks CD4 |
| GHOST | Human osteosarcoma cell | Expresses different co-receptors, adherent and well suited for ß-gal assays |
| PM-1 | Derivative of HUT 78, a neoplastic T-cell line | Expresses CD4, CXCR4 and CCR5 |
| MT-2 | HTLV-1 transformed | Expresses CXCR4, produces syncytia when infected with X4 isolates |
| C8166 | HTLV-1 transformed | Expresses CXCR4, produces syncytia when infected with X4 isolates |
| Peripheral blood mononuclear cells (PBLs) | Human whole blood | Contains a mixture of cell types infectable by different strains of HIV, similar to *in vivo* situation |

**Table 2.1:** characteristics of the different cell types used in this study

### 2.2.4 Biologica methods

#### 2.2.4.1 Ethical clearance for field study in Cameroon

In order to collect blood samples from HIV-infected or suspected individuals, an ethical clearance was obtained from the Cameroon Ministry of Public Health. The consent of the study subjects was also sought after which a questionnaire provided by the Ministry of Health was completed for each patient. Briefly, the questionnaire was composed of questions regarding the personal and social status (e.g. age, sex, marital status, occupation, area of residence) and also past and present health status of subjects.

#### 2.2.4.2 Sample collection

Five ml of whole blood was collected in vacutainer tubes containing anti-coagulants (EDTA; Becton-Dickinson) from individuals highly suspected or known to be infected with HIV in Limbe and Buea (South West Province; n=28), Bamenda (North West Province; n=10) and Yaounde (Centre Province; n=9). A larger sample (25 ml) was then collected from those who were positive by ELISA using “baby” blood bags containing the anticoagulant mixture CPDA-1 (citrate-phosphate-dextrose-adenosine).

#### 2.2.4.3 Serological testing by the enzyme-linked immunosorbent assay (ELISA)

Blood plasma was separated from cells of whole blood samples by centrifugation at 1000 rpm about an hour after blood collection. Plasma samples were screened for the presence of HIV antibodies using a commercial ELISA kit (Enzygnost HIV1+2, Dade-Behring). Within the well of a microtitre plate the patient`s antibody binds to antigen coated on the bottom of the plate. Patient`s antibody is subsequently detected with a peroxidase (POD)-conjugated second antibody (and antigen) after a washing step. Addition of a substrate enables the visualisation of an enzymatic action through the conversion of the colourless substrate to its coloured derivative which can be quantified photometrically.

The double antigen sandwich ELISA which recognises antibodies to HIV 1+2 is more sensitive in the early phase of seroconversion but has a lower cross-reactivity with antibodies directed against variant HIVs. The assay was performed based on the instructions of the manufacturers.

#### 2.2.4.4 Storage and transportation of samples

Blood samples were stored at 4°C for a maximum of 7 days, then packaged in plastic transport containers and transported to the Heinrich-Pette-Institut, Hamburg.

#### 2.2.4.5 Cell culture

Adherent cells were grown in DMEM supplemented with FCS, glutamine and antibiotics at 37°C in a 5 % $CO_2$ incubator. Suspension cells were grown in RPMI including the same supplements. To passage cells, adherent cells were washed with PBS from the plate surface, trypsinized and then further cultivated in fresh medium.

#### 2.2.4.6 Isolation of peripheral blood mononuclear cells (PBMC)

The Ficoll gradient density technique is used for isolating PBMC. Anti-coagulated blood is layered on Ficoll solution (Pharmacia Biotech, Uppsala, Sweden) and centrifuged enabling differential migration of different cell types (Boyum, 1968). Erythrocytes and granulocytes are found below the Ficoll solution while lymphocytes together with platelets and monocytes are recovered at the interface between plasma and the Ficoll layer. This layer is collected and washed with phosphate buffer saline to remove any platelets, Ficoll and plasma.
Anti-coagulated whole blood from either HIV negative or positive individuals was mixed 1:2 with PBS and carefully layered on Ficoll in 50 ml falcon tubes and the gradient centrifuged at 1200 rpm for 15 min in a table centrifuge at room temperature. Plasma was removed from the top of the gradient and stored in small aliquots at –20°C for further analysis. The mononuclear cells were then carefully removed and washed two times in PBS. This fraction contains about 95 % lymphocytes and is therefore termed peripheral blood lymphocytes (PBL). PBLs were cultured in 100 ml RPMI 1640 with 20 % FCS and stimulated with 0.5 µg PHA for 48 hours in a 5 % $CO_2$

humid incubator. On the third day, 20 U/ml of IL-2 was added to the cells and further cultivated for a day and then used for co-cultivation.

#### 2.2.4.7 Virus isolation

Since PBLs are the cells of choice for HIV recovery, they were isolated from whole blood samples collected from HIV infected individuals using the above method. After storing an aliquot of purified PBLs at –70°C, the remainder was co-cultivated with PM-1 cells and HIV negative PHA / IL-2 stimulated PBLs respectively. Both cultures were maintained by adding growth medium (RPMI 1640 with 10-20 % FCS, 2 mM glutamin and antibiotics) twice a week and fresh PBLs once a week to the PBL culture. Cultures were monitored daily for cytopathic effects (CPE) and syncytium formation under a microscope. Culture aliquots were collected twice weekly for p24 antigen production using antigen capture ELISA. Cultures positive on two consecutive occasions were harvested, centrifuged and the cell-free supernatants and pellets stored at -70°C for further analysis. The primary HIV-1-O isolate used in this study was kindly provided by Prof. Dr. Lutz Gürtler, University of Griefswald.

#### 2.2.4.8 Determination of virus growth by measuring p24 antigen production in primary cultures

Determination of virus growth in culture was by detection of p24 antigen production using an in-house p24 assay (Konvalinka *et al*., 1995). In this assay antibody instead of antigen is bound to the plate and therefore the antigen (capsid) present in the culture supernatant is captured. NUNC immuno plates were coated with 100 µl / well of Ascites 183 antibody and incubated overnight in a humid chamber at room temperature. Plates were then washed and blocked with 200 ml blocking buffer preventing unspecific binding and incubated for at least 2 hours at 37°C. After washing, 100 µl of appropriately diluted cell culture supernatant pre-treated with 0.1 % Triton-X-100 as well as serially diluted capsid standard were added in duplicate and incubated overnight. After washing, 100 µl of diluted rabbit-anti-capsid antibody (1:10,000) was added, incubated at 37°c for 1 hour, washed and 100 µl anti-rabbit POD (1:2000) added and incubated at 37°C. The substrate ($H_2O_2$) /chromogen (TMB) mixture, diluted 1:100 was added after a washing step and colour was allowed

to developed for 5-10 min and then stopped with 50 µl $H_2SO_4$. The OD was measured at a wavelength of 450 nm using a spectrophotometer.

### 2.2.4.9 Determination of the biological phenotype of viruses

MT-2 is an HTLV-1 transformed cell line used as a prognostic marker for disease progression during HIV-1 infection (Karlsson *et al.*, 1994). These cells express CXCR4 but not CCR5 co-receptor and can therefore not be infected by CCR5 isolates, while CXCR4 isolates which infect them produce syncytia. Cell-free virus supernatant containing 50 ng p24 antigen from HIV-1 primary cultures were used to infect $2x10^5$ MT-2 cells in a 12 well plate and incubated overnight at 37°C in a humid 5 % $CO_2$ incubator. Cells were then washed with PBS re-suspended in culture medium. Cultures were tested for capsid antigen production once a week and monitored daily for syncytium formation for 2 weeks using a microscope.

### 2.2.4.10 Co-receptor usage by viral isolates

GHOST cells are human osteosarcoma cells stably expressing either one of the human chemokine receptors (CCR1, CCR2b, CCR3b, CXCR4, CCR5, CCR8, BOB, Bonzo, or CX3CR1(V28) used by HIV strains for entry into the cell (Cecilia *et al*., 1998). They were plated at 5 x $10^3$ cells / well in 48 well plates and incubated overnight at 35°C in a humid 5 % $CO_2$ incubator. After removing culture supernatants, cell-free virus supernatant containing 50 ng of p24 antigen was then added and the culture incubated overnight. Fresh medium was added to a total volume of 500 ml / well and plates were incubated for 3 days at 37°C. Supernatant was removed and cells washed with PBS, fixed for 5 min with 500 µl methanol / acetone (1:1) and then washed with 1 % FCS in PBS and immunostained for intracellular p24 antigen as follows. 100 µl of the primary antibody (Ascites 183) diluted in PBS / FCS was added and incubated at 37°C for 1 hour and washed twice with PBS / FCS before adding 100 µl of the ß-galactosidase-conjugated secondary antibody. After incubation at 37°C for an hour, cells were washed with PBS / FCS and then PBS alone; and 100µl of X-gal (ß-galactosidase substrate) added and incubated at 37°C until colour developed. Determination of blue-stained focus

forming units (ffu) of infection representing cell to cell fusion, local viral spread and division of infected cells was by light microscopy.

### 2.2.4.11 Transfection of adherent cells with full-length molecular clones

Introduction of DNA into cells was done by the calcium phosphate precipitation method (Chen and Okayama, 1987). 1 x $10^6$ 293T or HeLa P4 cells were seeded on a 10 cm petridish and incubated overnight at 37°C. On the next day the medium was replaced with exactly 9 ml fresh medium and then incubated for a further 1-2 hours. 15 µg DNA (HIV full-length molecular clones) was added in 500 µl 0.25 M $CaCl_2$ solution after which it was mixed with a 500 µl 2 x BSS. After 15 min incubation at room temperature this mixture was introduced drop-wise onto the medium in the petridish containing the cells with gently swirling. The cells were then incubated overnight at 35°C in a humid 3 % $CO_2$ atmosphere incubator. On the next day the medium was replaced with new medium after washing the cells twice with PBS. Cells were then cultivated for two days at 37°C in a humid 5 % $CO_2$ incubator. Treatment of harvested cells and supernatant was dependent on the experiment to be performed. In each case the supernatant was filtered using a 0.45 µm filter and aliquots stored at –70°C for future use.

### 2.2.4.12 Infection of cells

Filtered supernatant obtained from transfection experiments were tested for the presence of p24 antigen. One ml of positive culture supernatants was used to infect 9 ml of 1x$10^6$ cells (PM-1 or PBLs) and incubated overnight at 37°C in a humid 5 % $CO_2$ incubator. Cells were then washed twice with PBS and re-suspended in RPMI 1640 and then cultivated for 2 weeks with p24 sampling done twice weekly. Positive supernatant were filtered and new cells re-infected and infectivity tested for two weeks. Any full-length clone that produced virus after at least two passages was termed “infectious”.

### 2.2.4.13 Preparation of virus stocks, virus titration and replication kinetics

Virus stocks of the infectious molecular clones and their parental isolates were generated by infecting PM-1 cells overnight, washing the cells twice and then further cultivation for 5 days at 37°C in a humid 5 % $CO_2$ incubator. The 50 % tissue culture infectious dose ($TCID_{50}$) was determined on either PM-1 or C8166 cells using 100 µl of serially diluted virus in quadruplicate in a 96 well plate by counting syncytia formation after cultivation for one week (Reed and Muench, 1938). To determine the replication kinetics of infectious molecular clones in relation to their parental isolates, 5 x $10^5$ PM-1 cells and 1 x $10^6$ PBMCs were respectively challenged with 500 $TCID_{50}$ and 1000 $TCID_{50}$ virus supernatant in a total volume of 1 ml in quadruplicate in a 48 well plate and incubated overnight at 37°C in a humid 5 % $CO_2$ incubator. The next day the cells were washed twice with PBS, re-suspended in 1 ml RPMI medium and maintained for 2 and 3 weeks respectively with p24 samples collected every 2-3 days. On days 5, 10 and 15 post-infection, cultures were fed by respectively replacing 500 µl of culture supernatant with fresh medium.

### 2.2.4.14 Electron microscopy

Virus from the molecular clones was generated by transfection of HeLa P4 cells and then passaged once on PM-1 cells. The primary isolates were also passaged once on PM-1 cells. When syncytia formation became evident, cells were collected by centrifugation at 400 g for 5 min and washed once with PBS. Using the protocol of Wiegers *et al*. (1999), cells were fixed with cold 2.5 % glutaraldehyde in 100 mM piperazine-N,N'-bis 2-ethanesulfonic acid (PIPES), pH 6.9 for 15 min at room temperature. They were then washed 3 times with cold PIPES, and post-fixed in 1 % osmium tetroxide in PIPES for 30 min at 4°C. After extensive washing in PIPES, cells were embedded in agarose (low gelling temperature; Sigma) and further treated with 1 % tannic acid for 10 min. Finally, agar blocks were washed in water, stained with 1 % uranyl acetate for 30 min in the cold, dehydrated in ethanol and embedded in ERL resin. Silver-grey sections were stained with lead citrate and uranyl acetate. Sections were examined under a Philips CM120 electron microscope at 60kV.

### 2.2.4.15 Isolation of genomic DNA from eukaryotic cells

To extract genomic DNA from infected eukaryotic cells either the DNAzol (Life Technologies, NY, USA) or the Qiagen kits were used, following the instructions of the manufacturers. DNA was extracted from about $1x10^7$ cells and quantified with a spectrophotometer. Depending on the type of PCR to be performed, between 50-250 ng DNA was used.

## 2.3 DNA molecular analysis

All DNA molecular analytical work was done based on the protocols of Sambrook *et al*. (1989) in appropriate laboratory containment facilities.

### 2.3.1 Materials for molecular analysis

### 2.3.2 Important chemicals, solutions, buffers, enzymes and kits

EDTA 0.5 M (pH 8.0)
Absolute ethanol
Ethanol 70 %
Isopropanol
Sodium acetate 3 M (pH 5.2)
Ammonium acetate 6 M
SDS 10 %
Tris HCl, 1 M, pH 8.0
TE buffer: 10 mM Tris HCl (pH 8.0) und 0.5 mM EDTA
Ethidiumbromide 1 µg/ml
Solutions for preparation of plasmid DNA (Maxi-Prep; Machery-Nagel, Düren)
Buffer S1: 50 mM Tris/HCl, 10 mM EDTA, 100 µg RNAse A / ml, pH 8.0
Buffer S2: 200 mM NaOH, 1 % SDS
Buffer S3: 2.80 M KAcO, pH 5.1
Buffer N1: 100 mM Tris/$H_3PO_4$, 15 % ethanol, 400 mM KCl, pH 6.3
Buffer N2: 100 mM Tris/$H_3PO_4$, 15 % ethanol, 900 mM KCl, pH 6.3
Buffer N3: 100 mM Tris/$H_3PO_4$, 15 % ethanol, 1150 mM KCl, pH 6.3

Buffer N5: 100 mM Tris/$H_3PO_4$, 15 % ethanol, 1000 mM KCl, pH 8.5
TENS Buffer: 1 M Tris pH 8.0, 0.5 M EDTA, pH 8.0, 5 N NaOH, 10 % SDS, 50 µg RNAse/ml
50X TAE buffer: Tris-base 2 M, acetic acid 0.25M, EDTA 0.05
10X TBE buffer: Tris-base 0.89 M, boric acid 0.89 M, EDTA 0.02 M
5X DNA loading buffer for agarose gel: 50 % glycerine, 20 mM Tris HCl (pH 8.0), 20 mM EDTA, 0.01 % bromo-phenol blue

Kits
DNAzol (Life Technologies, NY, USA)
Qiagen DNA isolation kit (Hilden)
Qiaquick PCR purification kit (Hilden)
Qiagen gel purification kit (Hilden)
Dye terminator cycle sequencing (DTCS) kit (Beckman Coulter, CA, USA)
AmpliTaq-Big Dye sequencing kit (Applied Biosystem, Foster City, CA, USA)
Sample loading solutions
Polyacrylamide gel (Beckman Coulter, CA, USA)

Restriction enzymes, T4-DNA ligase, Taq polymerase, Taq/Pfu DNA polymerase, RNAse A were from the following firms: New England Biolabs (Schwalbach / Taunus), Roche Diagnostics (Mannheim), Pharmacia Biotech (Freiburg), Stratagene (Heidelberg), MBI Fermentas (St. Leon-Roth) and Sigma (Deisenhofen), Molecular weight marker (New England Biolabs, Schwalbach / Taunus). All reagents were stored at –20°C.

### 2.3.3 Media and Reagents for bacteria culture

All media (Sambrook *et al.*, 1989) and solutions for bacteria cultures were either autoclaved or sterilely filtered immediately after production.
LB (Luria Bertani)-medium (“Broth”): trypton 10 g/l, yeast extract 5 g/l and NaCl 5 g/l
LB-plates: autoclaved LB medium with 1.5 % agar was cooled and then poured in sterile 10 cm petridishes and stored at 4°C.
LB-ampicillin or LB-kanamycin plates: LB-Agar plates with 100 µg/ml ampicillin or kanamycin added after cooling and stored at 4°C.

SOB-medium: 20 g/l trypton, 5 g/l yeast extract, 10 mM NaCl, 2.5 mM KCl. Shortly before use, 10 mM $MgCl_2$ was added.
Buffer TfB1: KacO 1.5 g, $MnCl_2$ 5 g, KCl 3,75 g, $CaCl_2$ 0.125 M, glycerol 75 ml, pH 5.8
Buffer TfB2: MOPS 1 g, $CaCl_2$ 0.25 M, KCl 0.375 g, glycerol pH 7.0
Ampicillin and kanamycin: 100 mg/ml in water, sterilised by filtration and stored at -20°C
X-gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactoside): 1% in dimethylformamide (DMF) stored protected from light at –20°C.

### 2.3.4 Molecular methods

### 2.3.5 Quick DNA preparation by the TENS method

For small scale plasmid DNA preparation, the TENS method was applied (Zhou *et al*., 1990). DNA preparation was carried out at room temperature. 1.5 ml of overnight culture was pelleted in an eppendorf tube by centrifugation in a microcentrifuge at 12.000 rpm for 15 sec. Pellet was re-suspended in 300µl TENS by mixing and then 150 µl of 3.0 M NaAc pH 5.2 added, mixed and spun for 7 min at full speed to pellet cell debris and chromosomal DNA. Supernatant was then poured into a fresh tube, precipitated with 0.9 ml of ice-cold absolute ethanol and centrifuged for 5 min at full speed to pellet DNA. Pellet was washed twice with 70 % ethanol, air dried and the DNA dissolved with 150 µl TE/ RNAse. This was then used for restriction digestion.

### 2.3.6 Preparation of large amounts of DNA by the alkaline lysis method

To prepare large quantities of plasmid DNA for transfection of cells and sequencing reactions, 200 ml of *E.coli* XL-1 blue overnight culture was prepared by the alkaline lysis method. This was done using buffers S1-S3 purified with the Nucleobond®ion exchange silica cartridges (Macherey-Nagel, Düren), washed with buffer N4 and eluted with buffer N5. Purified plasmid DNA was then precipitated with isopropanol and washed with 70 % ethanol. Dried DNA was re-dissolved in TE buffer giving a total concentration of 500-800 µg plasmid DNA that was used for further applications.

### 2.3.7 Ethanol DNA precipitation

Generally, DNA can be quantitatively precipitated with ethanol and re-dissolved in another buffer.
Ethanol precipitation: 0.1 volume 3 M NaAc and 2 volumes absolute ethanol were added to a given amount of DNA containing solution and incubated for 10 min at –20°C, and then pelleted in a microcentrifuge at –70°C for 10-15 min at full-speed. Obtained pellet was washed with 70 % ice cold ethanol, air dried and re-dissolved in a given volume of water or TE buffer
Isopropanol precipitation: 0.8 to 1.2 volume isopropanol was mixed with a given quantity of DNA depending on the salt concentration and then processed as described for ethanol precipitation.

### 2.3.8 Cleavage of DNA using restriction enzymes

Cleavage of plasmid DNA with restriction enzymes was done using standard methods according to the instructions of the manufacturers. The volume of the enzyme used was always less than 10 % of the total volume of the digest to prevent inhibition of the reaction.
A diagnostic digest was done using 1-2 µg DNA (5 µl miniprep DNA) in a total volume of 20 µl. For preparative purposes between 2-20 µg DNA (10-40 µl miniprep DNA) was used in a total volume of 50 to 100 µl. 45 µl of PCR fragments amplified with primers containing specific restriction sites were precipitated by mixing with 0.5 volume 6 M $NH_4Ac$ and 3 volume absolute ethanol, vortexed briefly and incubated for 30 min at –20°C. Pellets were obtained by centrifugation at full-speed for 20 min at 4°C, washed in 70 % ice cold ethanol, re-dissolved in water, incubated at 37°C for 30 min and then cleaved with a specified restriction enzyme.

### 2.3.9 Electrophoretic separation of DNA on agarose gel

A non-denaturing electrophoretic separation of DNA fragments was performed on a 0.7-1 % agarose horizontal gels with 2 µg/ml ethidiumbromide in TAE buffer at a voltage of 6-8 volt/cm. Before dispensing the samples on the gel they were mixed with 0.2 volumes loading buffer. A commercially available 1 kb marker was used to

determine fragment sizes. DNA fragments were made visible by the fluorescence of the intercalating EtBr with a UV-transiluminator (254 nm) and the results documented by a video detection sytem.

### 2.3.11 Isolation of DNA-fragments

After electrophoretic separation on the agarose gel, gel pieces with the desired DNA fragments were sliced out under long wavelength UV-light (366 nm). The DNA fragments were then extracted using the Qiagen gel extraction kit according to the instructions of the manufacturers. The principle is based on the fact that at 50°C in the presence of high concentration of chaotropic salts agarose easily melts. This mixture is then poured onto a spin column containing a silica membrane and spun at full speed. Under these conditions the DNA binds to the silica membrane while contaminants pass through the column. The impurities are efficiently washed away and the DNA is eluted from matrix with water or a buffer with low salt concentration.

### 2.3.12 The polymerase chain reaction (PCR)

The PCR method results in the selective amplification of a chosen region of a DNA molecule in the presence of specific oligonucleotides (primers) complementary to the DNA molecule and heat stable polymerase. Each PCR was performed in a total volume of 50 µl composed of the following: 250 nM dNTPs, 25-250 ng template DNA, 20 pmole/µl primer, 1 unit polymerase, PCR buffer: 20 mM Tris HCl pH 8.2, 5 mM KCl and 4 mM $MgCl_{2.}$

Enzymes used for PCR were chosen according to the length of the fragment to be amplified. Short fragment PCR of less than 1 kb were performed with *Taq* polymerase only, while “Long PCR” fragments which are greater than 3 kb were generated with the Expand High Fidelity PCR System (Roche, Mannheim) following the manufacturer’s instructions. This system is composed of a unique enzyme mix containing thermostable *Taq* and Pwo polymerases (Barnes *et al.*, 1994). Pwo (*Pfu*) polymerase has a 3`-5`exonuclease proof-reading activity therefore minimising the possibility of PCR based mutations introduced by *Taq* polymerase. PCR was done on a Peltier Thermal Cycler (PTC) 200 with heated lid (MJ Research, MA, USA) and specific cycling conditions (see appendix) based on the fragment length, hybidisation

strength of primers and type(s) of polymerase used. Amplified products were either directly purified by the Qiaquick DNA purification method or gel purification (Qiagen, Hilden).

### 2.3.13 Amplification of PCR fragments and generation of full-length molecular clones of HIV-1

Short fragment nested PCR was used to amplify selected regions (650-750 bp) of the HIV genome which were later used for genetic sub-typing. The long PCR approach was used to amplify the entire HIV proviral genome in two fragments. This was necessary due to the redundancy of the LTRs.

### 2.3.14 Oligonucleotides (primers)

Oligonucleotides were ordered either from the research group of Dr. Michael Schreiber at the Bernhard-Nocht-Insitute for Tropical Medicine, Hamburg or from Metabion, a commercial firm in Munich. The list of primers used is found in the appendix. Oligonucleotides used for short fragment PCR were designed to bind to conserved regions within the genes amplified. Primers used for long PCR included restriction sites which later facilitated the cloning of these fragments into vectors.

### 2.3.15 Ligation of DNA fragments into vectors

#### 2.3.15.1 TA cloning vector

*Taq* polymerase has a non-template dependent terminal transferase activity which adds a single deoxyadenosine (A) to the 3`ends of PCR products. This facilitates the cloning of *Taq* amplified fragments into a commercially available linearized TA vector pCR®-XL-TOPO® (Invitrogen, The Netherlands) This vector has single overhanging deoxythymidine (T) residues at the 3' ends. By exploiting the ligation activity of topoisomerase (Shuman, 1994), ligation to PCR amplified DNA occurs within 5 min at room temperature. Between 5 and 40 ng DNA was ligated into 1 ng of linearised vector. Reaction was stopped after 5 min and 10 µl ligation reaction was then used to transform super-competent bacteria provided with the kit.

### 2.3.15.2 Cloning into a circularised vector

PCR fragments amplified with primers containing restriction sites or DNA fragments from other plasmids were cleaved, gel purified and cloned into a circularised vector (bluescript; Stratagene) cleaved open with a similar enzyme combination. Between 5 and 50 ng vector were ligated with DNA fragments in a 1:5 molar ratio and in a total volume of 20 µl with 3 units T4 DNA ligase and incubated at 16°C overnight. In all ligation experiments, a control reaction composed of vector only was included. Competent bacteria were transformed with 10-20 µl ligation reaction.

### 2.3.16 DNA sequencing reaction

Sequencing reactions were carried out using the chain termination method (Sanger and Coulson, 1979). In this method 2',3' dideoxynucleotide triphosphates (ddNTP) which differ from conventional dNTPs in that they lack a hydroxyl residue at the 3' position of deoxyribose are used. When incorporated into a growing DNA chain they prevent the formation of a phosphodiester bond with a succeeding dNTP since they lack the 3' hydroxyl residue. Recent improvements in sequencing methods have led to the replacement of radioactive labels with fluorophores enabling "one tube" reactions and also the automation of sequencing procedures.
Reactions were done using between 50-500 ng double stranded PCR product or purified plasmid DNA derived from maxi preps depending on the kit used. For nested PCR products which were directly sequenced, the second round PCR inner primers were used, while for full-length clones, the "primer walking" approach was implemented. In each case the reaction was composed of 4 pmol sequencing primer in a total volume of 20 µl including, 2 µl BigDye-buffer and 6 µl Half-Term buffer (AmpliTaq-BigDye sequencing kit, Applied Biosystem, Foster City, CA, USA) or 12 µl of master mix from the Dye terminator cycle sequencing (DTCS) kit (Beckman Coulter, CA, U.S.A.). The PCR cycle sequencing conditions were: 96°C for 20 sec, 50°C for 11 sec and 60°C for 4 min for 25 cycles. Cycle sequencing products were precipitated in a solution containing 2 volumes 3 M NaAc pH 4.6, 2 volumes 100 mM EDTA and 1 volume glycogen (Roche, Mannheim). The pellet was washed once with absolute ethanol and then with 70 % ethanol, air-dried and re-dissolved in water or sample loading solution provided with the kit. The AmpliTaq-Big Dye system

sequencing which generated readable sequences of about 650 nucleotides was done on an ABI model 377A automated sequencer (Applied Biosystems, Foster City, CA, USA) at the Research group of Prof. Dr. Dietmar Richter at the Centre for Molecular Neurobiology, Hamburg; while the automated CEQ 2000 capillary sequencer (Beckman Coulter CA, USA) which generates sequences of about 450 nucleotides was used to sequence products prepared using the DTCS kit at the Department of Virology, University of Heidelberg.

### 2.3.17 Computer-aided analysis of DNA sequences

#### 2.3.17.1 Phylogenetic analyses

Contigs of full-length sequences were assembled with the Contig Assembler of the Vector NTI Suite (InforMax, Oxford, UK). Sequences of the gene fragments were aligned automatically using CLUSTAL X (Thompson *et al.*, 1997) with minor manual Adjustments using the Sequence Alignment Editor (SeAl) (Rambaut, Oxford, UK) taking into consideration the protein coding sequences. The new sequences were aligned with the recent compilation of non-recombinant and Circulating Recombinant Forms (CRF) HIV-1 sequences from the HIV Los Alamos database. Phylogenetic analyses of these sequences were performed using the neighbour joining method of the CLUSTAL X program. During the final analysis, gaps that were introduced in order to create the alignment were stripped. The statistical robustness of the neighbour-joining tree and reliability of the branching pattern was confirmed by bootstrapping (1000 replicates). Only bootstrap values greater than 70 % were considered statistically significant. To prevent the possibility of new sequences obscuring the genetic relationships, unknown sequences were phylogenetically analysed one at a time. To determine possible inter-subtype recombinants and recombination events, the SIMPLOT version 2.5 was used to perform Similarity and Bootscanning analyses (Ray, 1999) in a sliding window of 300 bases with 30 bp increments or 500 bases with 100 bp increments. Similarity analysis compares recombinant sequences to a number of reference sequences representing different subtypes defined by the user and calculates the percentage similarity of the query sequence with respect to the reference sequence. The Bootscanning method (Salminen *et al.*, 1995a) of this program maps breakpoints within a genome with high

accuracy. The query sequence is analysed with the supposed parental sequences and an out-group reference subtype. Different parts of recombinant genomes belong to different subtypes with the crossover point(s) in each recombinant group defined by the recombination event(s). This was confirmed by phylogenetically analysing various fragments based on the breakpoints in the mosaic genome.

#### 2.3.17.2 Drug resistance analysis

Putative protease and reverse tanscriptase drug resistance mutations were determined using the Anti-viral Drug Resistance (ADRA) tool from the HIV-1 database (http://hiv-web.lanl.gov). Codons 9-99 of protease and 1-130 of reverse transcriptase were analysed. The ADRA tool compares the input (query) sequences to known sequences of the HIV-1 B subtype isolates shown to confer resistance to anti-HIV drugs.

### 2.3.18 Bacteria strains

The *Escherichia coli (E.coli)* strain XL-1 Blue (Stratagene) of the genotype: *hsd*R17, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*[F´, *pro*AB, *lac*$I^q$Z∆M15, Tn10, ($Tet^r$)] was used in most cloning experiments. The advantage of this bacteria strain is that it is highly transformable and also has a low recombination rate.
For transformation experiments with the pCR®-XL-TOPO® vector (Invitrogen) “super competent” bacteria of the genotype [$F^-$*mcr* A∆(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*Z∆M15 ∆*lac*X74 *rec*A1 *deo*R *ara*D139 ∆(*ara-leu*) 7697 *gal*U *gal*K *rsp*L ($Str^R$) *end*A1 *nup*G] supplied with the Cloning kit were used.

### 2.3.19 Production of transformation competent bacteria

Highly competent bacteria were prepared according to the protocol of Inoue *et al.* (1990). 250 ml of SOB-medium was inculated with 2 ml *E.coli* overnight culture and incubated at 37°C until it reached an $OD_{600}$ of 0.5. The bacteria culture was kept cold on ice, centrifuged, re-suspended with 20 ml cold TfB1 and incubated for 10 min on ice. This was followed by centrifugation and the pellet obtained was re-suspended in 5 ml buffer TfB2, aliquoted in 100 µl and then shock frozen in liquid nitrogen before

being stored at –80°C until used. With this method a transformation efficiency of up to $10^7$ colonies / µg plasmid DNA was obtained.

### 2.3.20 Bacteria transformation

Transformation competent *E.coli* bacteria were thawed on ice and 50 µl bacteria suspension was inoculated with 10-20 µl DNA (mostly a ligation reaction), mixed and incubated on ice for 30 min. The bacteria were then exposed to a “heat shock” at 42°C for 30 sec in a water bath, incubated for a further 2 min on ice after which 250 µl LB-medium was added and further incubated at 37°C for 60 min in order to allow the expression of the selectable marker gene (e.g. ß-lactamase enzyme in the case of ampicillin resistant plasmid). The total volume of the transformation reaction was then plated on an ampicillin or kanamycin resistant agar plate and incubated at 37°C overnight.

### 2.3.21 Liquid bacteria culture

Culturing of transformed bacteria was always done in the presence of the appropriate antibiotic (ampicillin or kanamycin). Small DNA cultures were prepared by inoculating 5 ml of LB-medium containing antibiotic with bacteria colony picked from the agar plate, and then incubated in a shaker (150 rpm) overnight at 37°C. For larger DNA cultures 200 ml LB-medium was inoculated in an Erlenmeyer flask with similar conditions as described above.

## 2.4. Biochemical analysis

### 2.4.1 Reagents for biochemical analysis

#### 2.4.1.1 Gel electrophoresis

Running buffer for protein gel: glycine 0.384 M, Tris-base 0.05 M, SDS 0.1 %
2 X loading buffer for protein gel: 2 % SDS, 2 % beta-mercaptoethanol, 0.118 M stacking gel buffer, 20 % glycerine
10 X stacking gel buffer: 0.47 M Tris HCl pH 6.7

Fixing and colour solution for protein gel: methanol 50 %, acetic acid 30 %, Coomassie-blue-R 0.1 %
Decolourising solution for protein gel: methanol 30 %, acetic acid 1 %
Sucrose cushion: 20 % (w/v) in water

#### 2.4.1.2 Immunoblot analysis

Western blot transfer buffer:
Buffer I: 0.3 M Tris, 20 % methanol
BufferII: 0.025 M Tris, 20 % methanol
BufferIII: 0.025 M Tris, 20 % methanol, 0.03 % DL-norleucine
Western blot blocking buffer: 10 % milk powder / 0.5 % Triton-X 100 in PBS
Western blot wash buffer: PBS with 0.5 % Triton-X 100
Western blot chromogenic substrates
BCIP (5-bromo-4-chloro-3-indolyl-phosphate) solution: 7.5 mg / ml in 100 % dimethyl formamide, stored at –20°C.
NBT (4-nitrotetrazolium-chloride blue) solution: 12.5 mg / ml in 70 % dimethylformamide, stored at –20°C.
Western blot reaction buffer
50 mM Glycin pH 9.6 (adjusted with NaOH) 40 mM $MgCl_2$
Low molecular weight (LMW) marker for SDS gel electrophoresis (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA)
New LAV Blot I confirmatory kit for the detection of human anti-HIV-1 antibodies in plasma / serum (Bio-Rad, Marnes la Coquette, France).

#### 2.4.1.3 Antibodies

Table 3.2 shows a list of primary and secondary antibodies used and their respective origins. The dilution of each antibody used for Western blot and detection by means of peroxidase or alkaline phosphatase is also given. The human antisera against HIV-1-M and O were obtained from HIV infected individuals and were diluted 1:500 before use.

| Specificity | Origin / species | Source | WB-dilution |
|---|---|---|---|
| α-HIV-1-NC (p7) | rabbit | Prof. Dr. Kräusslich | 1:2000 |
| α-HIV-1 MA (p17) | rabbit | Prof. Dr. Kräusslich | 1:5000 |
| α-HIV-1 CA (p24) | rabbit | Prof. Dr. Kräusslich | 1:10000 |
| α-HIV-1-IN (p32) | rabbit | Prof. Dr. Kräusslich | 1:2000 |
| α-HIV-1-RT (p51/66) | rabbit | Prof. Dr. Kräusslich | 1:5000 |
| Alkaline phosphatase conjugated α-rabbit | goat | Dianova, Hamburg | 1:5000 |
| Peroxidase-conjugated α-human | rabbit | Dako, Denmark | 1:1000 |

**Table 3.2:** Antibodies used in this study.

### 2.4.2 Biochemical methods

#### 2.4.2.1 Antigen (virus) preparation

The different strains of HIV used for Western blot were grown to high titre by infecting 1 x $10^7$ MT-4 cells in a T-175 flask and maintained daily by replacing a portion of the medium. When infectious titre had reached its peak (after about 7 days), culture was harvested and centrifuged at 3000 rpm for 5 min and the supernatant carefully laid on 20 % sucrose cushion and spun at 35000 rpm for 90 min in an ultra centrifuge using an SW40 rotor (Beckman). The pellet obtained was re-suspended in PBS and used for Western blot.

#### 2.4.2.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein analysis was done using the discontinuous SDS-gel electrophoresis (Laemmli, 1970) system in which proteins were separated based on their size; a 17.5 % resolving gel (acrylamide to bisacrylamide ratio of 30:0.8) and a 6.3 % stacking gel (acrylamide to bisacrylamide ratio of 40:1). Before loading the samples they were boiled with the protein loading buffer for 5 min. The mini-gel (9 x 7 x 0.15 cm; system 201, Hoefer, San Francisco, CA, USA) was used and the electrophoresis run at a constant current (20 mA for the stacking gel and 40 mA for the resolving gel).

#### 2.4.2.3 Immunoblot / Western blot (WB) analysis

After electrophoretic separation on the SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane (Schleicher und Schüll, Dassel) with a semi-dry Trans-Blot Apparatus (cti GmbH, Idstein,) at 0.8 mA per $cm^2$ for 2 hours at 4°C (Towbin *et al.*, 1979). The efficiency of this transfer was determined by staining with Ponceau-S (Sigma). To test for patient antibody reactivity against different viral proteins, nitrocellulose was cut into 0.3 cm wide strips. After decolourising with PBS, unspecific protein binding was blocked by incubation in 10 % milk powder / 0.5 % Triton-X 100 in PBS. Specific antibody was diluted in 10 % NCS in PBS and incubated overnight on a shaker at room temperature. After washing it twice, respectively for 15 min with PBS / 0.5 % Triton-X 100, the nitrocellulose was then incubated with the enzyme conjugated antibody for 2-3 hours at room temperature. The nitrocellulose was intensively washed thrice for 30 min each with PBS-Triton X-100 (0.5 %) and then with water for 5 min. The nitrocellulose was incubated with chromogenic substrate for 5 min during which the bands developed and the reaction was stopped with 0.5 % SDS in $ddH_2O$.

### 2.4.7 Correlation of in-house Western blot results with a standard commercial assay

To confirm serological results obtained by ELISA, a commercial immunoblot (Bio-Rad, Marnes la Coquette, France) was used. The assay uses the indirect ELISA technique on a nitrocellulose strip containing all the constitutive proteins of HIV-1 and an anti-IgG internal control. The assay was carried out based on the instructions of the manufacturers. Results obtained were compared with those of the in-house Western blot.

# 3. RESULTS

## 3.1. Characterisation of Primary Isolates of HIV-1

### 3.1.1. Serological Analysis

Reliable diagnosis of HIV requires the application of at least one of several methods: the enzyme-linked immunosorbent assay (ELISA), Western blot, virus isolation, indirect immunofluorescent assay and in recent times the polymerase chain reaction (PCR). Implementation of at least two different methods is necessary since emerging HIV variants may be undetected by any one of the currently used assays. Screening of blood samples for HIV antibodies with an ELISA followed by confirmation with a Western blot is the recommended procedure (WHO, 1987).

In this sudy, 47 blood samples were collected from four different locations across Cameroon (fig. 1.5B): Bamenda (n= 10) in the North West Province, Limbe (n= 26) and Buea (n= 2) in the South West Province, Yaounde (n= 9) in the Centre Province and screened by a third generation sandwich ELISA. This assay which is highly specific and sensitive, incorporates peptides of HIV-1-M, -O and HIV-2. Of the 47 samples, 45 were positive for antibodies to HIV, while two were negative although the subjects presented with typical signs and symptoms of AIDS. Infected individuals included tuberculosis patients and those presenting with known signs and symptoms of AIDS.

The reactivity pattern of the Cameroonian HIV samples in Western blots derived purely from primary virus material originating from Cameroon was compared to those made from subtype B viruses available commercially. The in-house Western blot strips were prepared from the 99CMBD6 isolate which had been obtained in this study. This was done because it may be anticipated that it could show better reactivity than commercial WB strips based on B-type HIV isolates. However reactivity of sera with these WB strips prepared from the Cameroonian isolate was weak. On the other hand, all sera were positive when screened against the commercial WB used for diagnostic purposes (fig. 3.1). The positive sera correspond to subtypes A, G, F2, CRF02.AG and HIV-1 group O. The sample 99CMLB40 obtained from an individual with AIDS had been initially found to be negative when

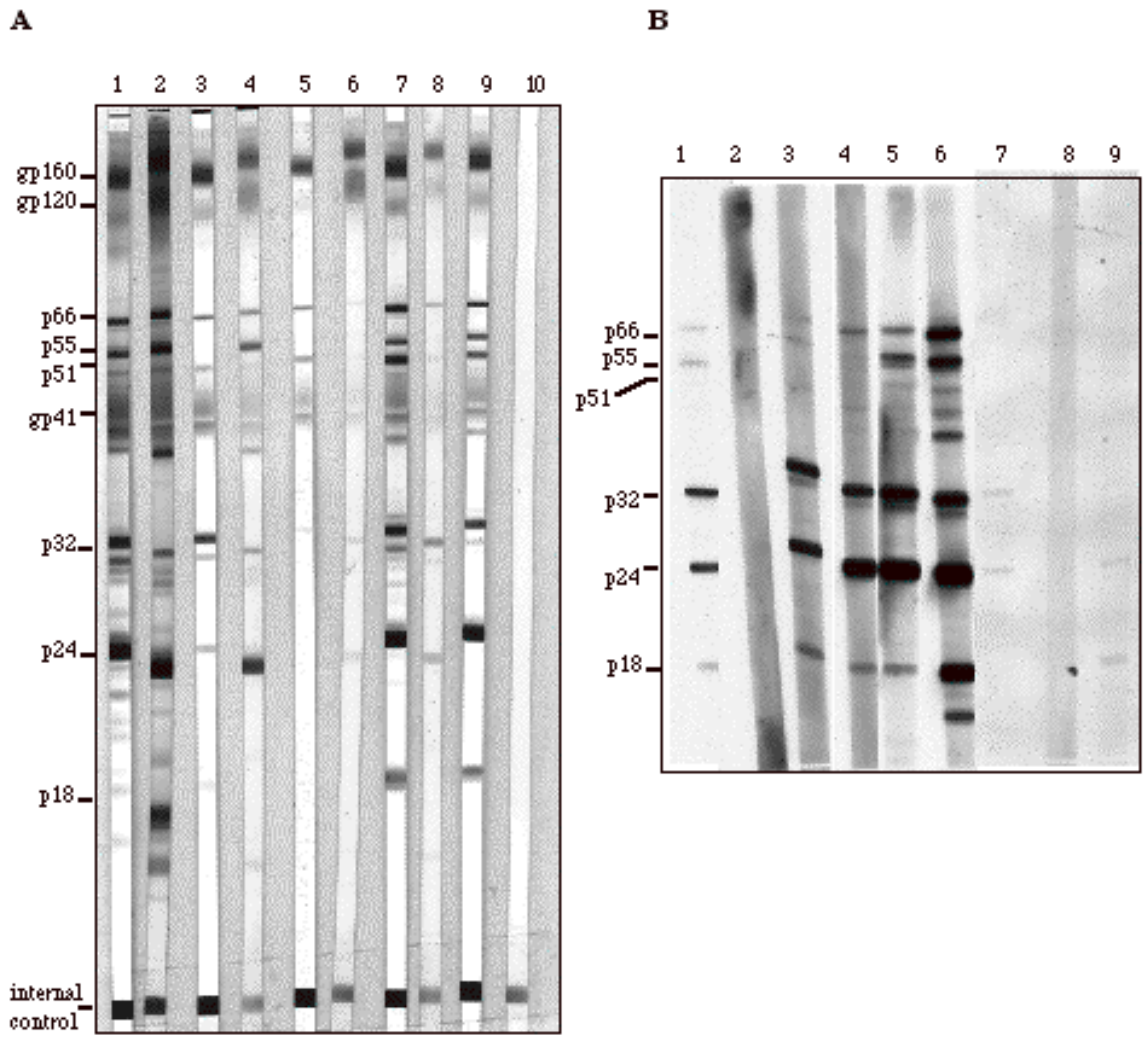


**Fig. 3.1:** Western blot analysis of selected Cameroonian HIV positive samples. The sizes of the different viral proteins are indicated on the left. The identification of each sample represented by numbers is at the top. A) Commercial WB: strip 1=99CMBD6; 2=99CMLB6; 3=99CMYD3; 4=99CMLB4; 5=99CMLB40; 6=99CMLB33; 7=subtype B; 8=group O; 9=Positive control; 10= Negative control. The envelope bands are seen as diffuse bands. 99CMLB40 (strip 5) does not show any reactivity to gag (p55, p24, p18). Its reactivity is even weaker than seen for the HIV-1-O sera. B) In-house WB strips derived from the isolate 99CMBD6; sample identification: lane 1=subtype B, 2= negative control, 3=group O; 4=99CMBD6; 5=99CMLB4; 6=99CMLB6; 7=99CMLB33; 8=99CMLB40; 9=99CMYD3. Reactivity of patient sera was very weak when compared to the commercial assay in A. Only gag and pol proteins could be detected. The sample 99CMLB40 (lane 8) was completely negative in this assay.

tested with a rapid HIV detection assay, but was positive in the commercial sandwich ELISA. When analysed with the commercial Western blot, this sample was reactive to envelope proteins (gp41, gp120 and gp160), and showed weak reactivity to pol (p32, p51 and p66), but lacked antibodies to gag protein (fig. 3.1A). Using the in-house WB assay, this sample was completely negative, showing lack of antibodies to

any of the known HIV proteins (fig. 3.1B). The WHO recommendation for HIV positivity by WB is the detection of at least two envelope bands with or without one or more bands of gag and/or pol on the WB strip. Based on the commercial WB results, the patient 99CMLB40 was classified as positive. Later molecular analysis showed that she carried an AJU complex recombinant HIV-1 strain (section 3.1.3.2). Using the in-house WB strips derived from the Cameroonian isolate 99CMBD6, it was not possible to detect envelope antibodies of any of the 45 ELISA positive patients even though antibodies to gag and pol could be readily detected (fig. 3.1B). This lack of detection could be as a result of the possible loss of envelope proteins during the process of virus preparation. It has also been observed that retroviral glycoproteins are more easily detected by radio-immuno precipitation rather than Western blot (Herchenröder, personal communication). Recently a plasma sample from an individual with clinical signs of AIDS from Benin in West Africa was found to escape detection by certain commercial ELISAs, however, molecular studies classified it as an AGJ recombinant (Baldrich-Rubio *et al*., 2001).

### 3.1.2. Virus Isolation

Most *in vitro* experiments to study HIV biology conducted to date use laboratory adapted HIV-1 strains which have undergone numerous rounds of expansion on immortalised cell lines which can result in loss and/or alteration of important viral features. Primary isolates, on the other hand, represent a valuable tool for phenotypic studies since they have not been passaged in cell lines and represent the virus population which directs infection at a given time point of disease. Such primary isolates are obtained by co-cultivation of patient derived PBMCs with those of HIV negative donors leading to isolation and expansion of related but distinct viral quasispecies that are present in an infected individual at a given point in time. However, the procedure of virus isolation is at times limiting because it may select for certain variants from the population of HIV-1 present in patient PBMC *in vivo* (Meyerhans *et al*., 1989). This not withstanding, biological analysis with virus supernatant from such cultures is representative of the dominant population found in the patient PBMC.

Primary isolates were derived from 19 of the 47 blood samples obtained from HIV infected individuals from the 3 provinces. Eighteen isolates were obtained by co-

culture with uninfected PBMC and 1 by co-culture with PM-1 cells. PM-1 is a permanent T-cell line that expresses CXCR4 and CCR5, the two main co-receptors required by HIV for infection (Lusso *et al*., 1995). The epidemiological and clinical data of the respective patients and samples are given in table 3.1. The cultivation time until first detection of HIV-1 capsid antigen was highly dependent on the patients' clinical status and isolates from AIDS patients were obtained within a shorter time (table 3.1). It has been observed that HIV isolates from patients at later stages of AIDS usually grow faster *in vitro* producing higher titre of capsid antigen (Tersmette *et al*., 1989). Although the reason for the inability to isolate virus from the other 28 samples could not be clearly determined, the long period between sampling and culturing and also the transportation conditions with probable shifts in temperature could account for this. Under these circumstances however, the 19 isolates obtained could be considered a relative success rate when compared to the analogous control experiments. As controls, HIV-1 isolation trials were performed with blood samples from five HIV positive individuals without any clinical signs and no history of anti-HIV-treatment. Lymphocytes were separated on the same day of blood collection and co-cultivated with HIV negative donor lymphocytes. Virus was isolated from two of the five blood samples collected, indicating a 40 % isolation rate.

### 3.1.3. Molecular Characterisation

#### 3.1.3.1. Phylogenetic analysis

To extend the knowledge on the distribution of various HIV-1 subtypes that are circulating in Cameroon as a whole and Western Cameroon in particular, discontinuous fragments from the 3 major structural genes *gag*, *pol,* and *env* of each HIV isolate was amplified using a nested PCR approach. Products obtained were gel purified and directly sequenced thereby providing a representation of the dominant pool of variants present in the primary isolate. These sequences were then aligned with reference subtypes and then phylogenetically analysed using appropriate software packages. Fig. 3.2 illustrates the genomic regions studied. A summary of the genetic characteristics of these isolates is represented in table 3.1.

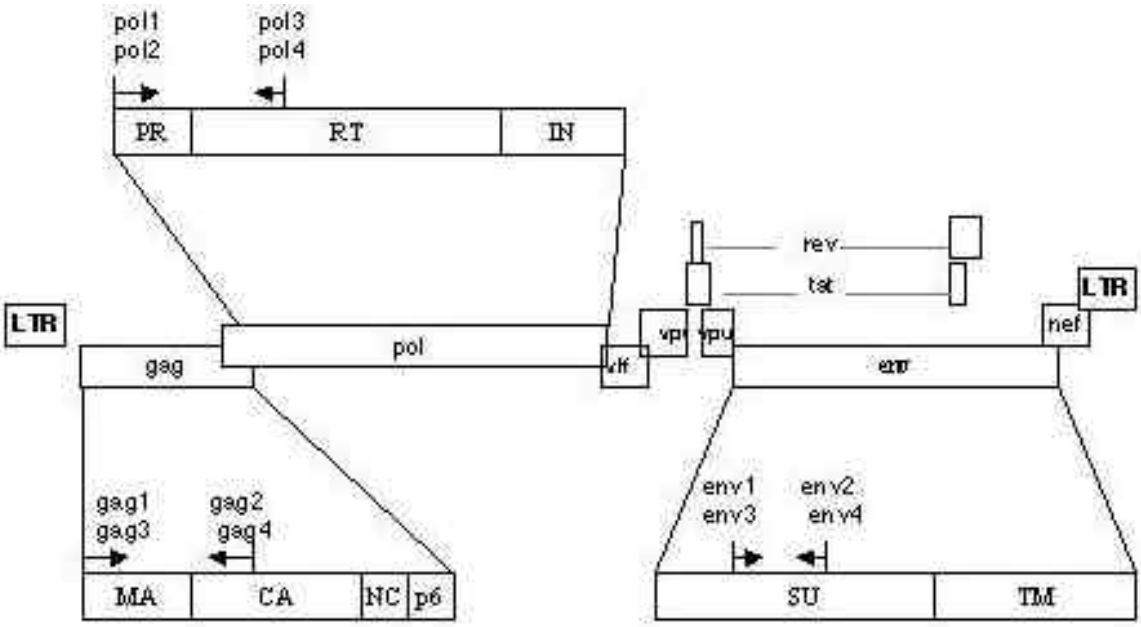


**Fig. 3.2:** Genomic structure of HIV-1 showing the various regions (enlarged) that were amplified and sequenced. The primers used for nested PCR and sequencing are indicated at the top of the arrows. The inner nested primers were also used for sequencing. Details about these primers and their other characteristics are indicated in the appendix. The diagram is not drawn to scale.

#### 3.1.3.1.1 Gag sequences

Fig. 3.3A shows a *gag* neighbour-joining tree of the new isolates (boxed) compared with HIV-1 reference sequences. The new sequences were distributed among subtypes A, G and F2. Generally, there are three different subtype A sequence clusters in *gag*. These represent “pure” subtype As, the CRF01.AE viruses mainly from Thailand and Central African Republic and the CRF.02 AG viruses predominantly from West and Central Africa. Due to the fragment length (~700bp) of the various genes analysed, distinction of CRF02.AG-like viruses from the others was only possible by looking at individual sequences. In the case of *gag*, of the 15 isolates that clustered with subtype A, eight of them sub-clustered with the reference CRF02.AG strains with a bootstrap value >70% (Howard and Rasheed, 1996). The isolates 99CMLB4, 99CMLB25 and 99CMLB26 which formed a loose cluster with the CRF01.AE strains were found not to belong to this group (bootstrap value <50%) when analysed individually, but to the group of pure subtype A *gag*. Three isolates clustered among the reference G strains while one was found to be of the newly described sub-subtype F2.

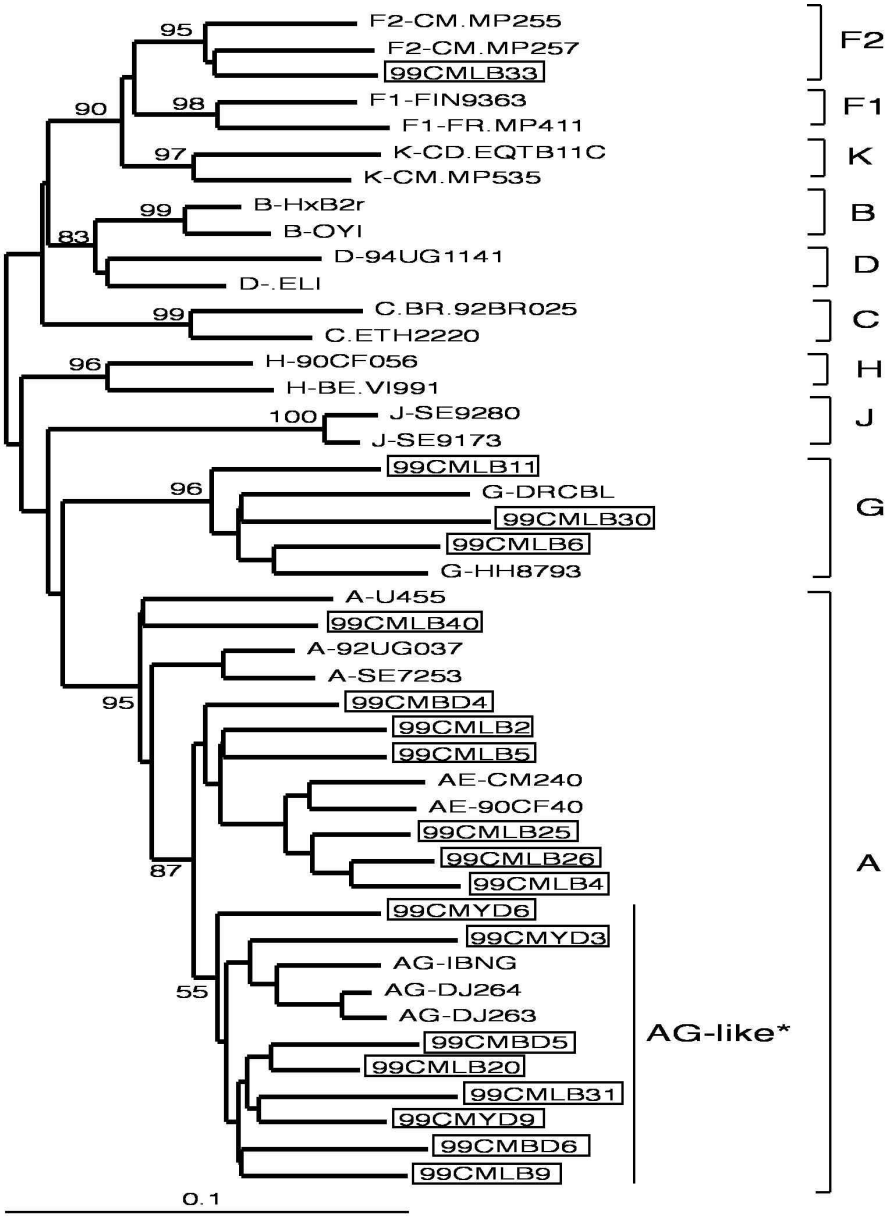


**Fig. 3.3A:** Phylogenetic analysis of *gag* sequences of the 19 HIV-1 primary isolates from Western Cameroon (boxed). The tree was generated using the neighbour joining method of the CLUSTAL X program. Horizontal branch lengths are drawn to scale but vertical branch lengths are for clarity only. Reference sequences were obtained from the Los Alamos data base. Subtypes are indicated to the right. Values at the nodes indicate the percentage bootstraps (1000 replicates) in which the cluster to the right was supported. Sequences marked AG-like* clustered with CRF02.AG viruses with bootstrap values >70%. The tree was rooted by using SIVcpzGab as an out group.

**Table 3.1:** Epidemiological, clinical, genetic and biological characteristics of Western Cameroonian HIV-1 primary isolates

| Isolate[a] | Age (yrs.) | Sex | Clinical status[b] | Geographic location | Cult. onset[c] | Antigen detection[d] | Genetic subtype[e] *gag* | *pol* | *env* | Biological phenotype | Co-receptor usage CCR1 | CCR3 | CCR5 | CXCR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 99CMBD4 | 47 | M | ARC | Bamenda | 7 | 21 | A | AG | AG | NSI | - | - | +++ | - |
| 99CMLB2 | 16 | F | ARC+tb | Limbe | 7 | 14 | A | AG | AG | NSI | - | - | +++ | - |
| 99CMLB4 | 18 | F | ARC+tb | Limbe | 7 | 29 | A | A | A | NSI | - | - | +++ | - |
| 99CMLB5 | 22 | F | ARC+tb | Limbe | 7 | 29 | A | A | A | NSI | - | - | +++ | - |
| 99CMLB6 | 31 | F | ARC+tb | Limbe | 6 | 29 | G | G | G | NSI | - | - | +++ | - |
| 99CMLB9 | 28 | F | asm+tb | Limbe | 6 | 16 | AG | AG | AG | NSI | - | - | +++ | - |
| 99CMLB11 | 46 | M | ARC+tb | Limbe | 6 | 16 | G | G | G | NSI | - | - | +++ | - |
| 99CMLB20 | 28 | M | ARC+tb | Limbe | 6 | 16 | AG | U | AG | NSI | - | - | +++ | - |
| 99CMLB25 | 37 | M | asm+tb | Limbe | 6 | 49 | A | A | A | NSI | - | - | +++ | - |
| 99CMLB26 | 31 | M | ARC | Limbe | 6 | 34 | A | A | A | NSI | - | - | +++ | - |
| 99CMLB31 | 32 | M | ARC | Limbe | 5 | 35 | AG | AG | AG | NSI | - | - | +++ | - |
| 99CMYD6 | 35 | F | ARC+tb | Yaounde | 1 | 21 | AG | A | AG | NSI | - | - | +++ | - |
| 99CMLB40 | 30 | F | AIDS | Buea | 5 | 16 | A | J | U | NSI | - | + | +++ | - |
| 99CMBD5 | 20 | F | AIDS | Bamenda | 7 | 21 | AG | AG | AG | SI | + | - | - | +++ |
| 99CMBD6 | 32 | F | AIDS | Bamenda | 7 | 15 | AG | AG | AG | SI | - | - | - | +++ |
| 99CMLB30 | 40 | M | ARC | Limbe | 5 | 15 | G | G | G | SI | + | - | - | +++ |
| 99CMLB33 | 45 | M | AIDS | Limbe | 1 | 15 | F2 | F2 | F2 | SI | - | - | - | +++ |
| 99CMYD3 | 33 | M | AIDS+tb | Yaounde | 3 | 9 | AG | AG | AG | SI | ++ | + | +++ | +++ |
| 99CMYD9 | 34 | M | AIDS+tb | Yaounde | 4 | 7 | AG | AG | AG | SI | - | - | - | +++ |

[a]Isolate names are preceeded by the year of collection, CM-Cameroon, F = Female, M = Male, [b]AIDS = Acquired immune deficiency syndrome, ARC = AIDS related complex, asm = Asymptomatic, tb = Tuberculosis, [c]Culture onset = Days post collection of blood sample, [d]Days post co-cultivation, [e]The “AG“ subtype classification is based on analysis of individual sequences (see section 3.1.3.1), NSI = Non-syncytium inducing isolate (slow / low), SI = Syncytium inducing isolate (rapid / high), + = <40 ffu/ml, ++ = 40-80 ffu/ ml, +++ = >80 ffu/ml (main co-receptor), ffu = focus forming unit

### 3.1.3.1.2 Pol sequences

*Pol* is the most conserved gene in all retroviruses. This property has led to its wide use for phylogenetic classification of lentiviruses (Gojobori *et al*., 1990). Until recently when HIV-1 drug resistance studies involving protease and RT became common, very few *pol* sequences were available in the HIV database. The sequences from the

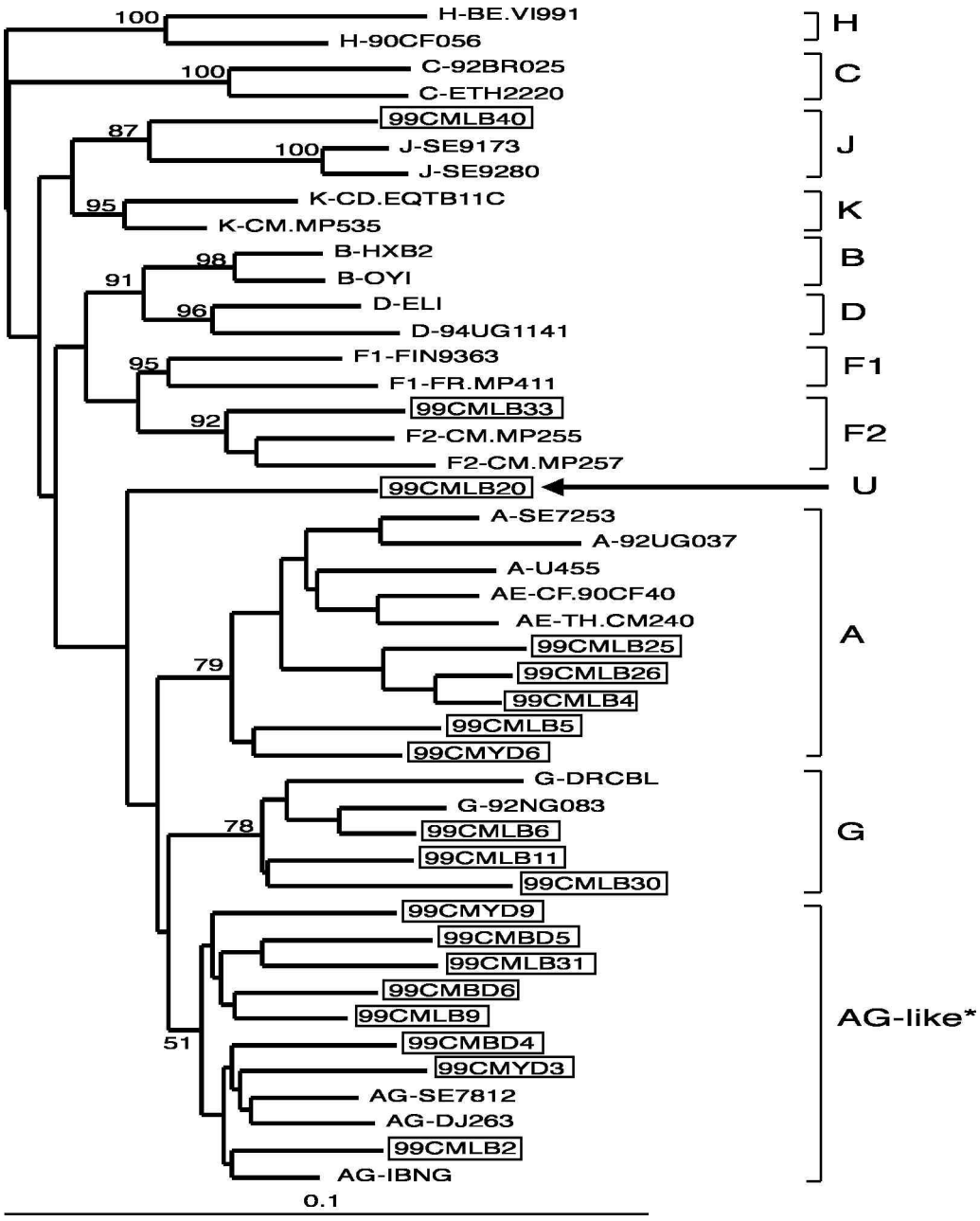


**Fig. 3.3B:** Phylogenetic tree constructed using *pol* sequences of the Western Cameroonian HIV-1 isolates (boxed). The tree was constructed as described for fig. 3.3A. The unclassified isolate 99CMLB20 is indicated as U (arrow). As observed for *gag* and *env*, AG-like sequences were the most dominant. The 99CMLB40 sequence clusters with subtype J sequences.

protease and 5' region of reverse transcriptase of the 19 isolates were classified into subtypes: A, CRF02.AG, G, F2 and J (fig. 3.3B). Five isolates clustered with subtype A, eight were CRFAG.02-like viruses, while one isolate (99CMLB20) did not cluster with any known subtype and was therefore termed “unclassifiable”. This refers to sequences which do not cluster with a statistically significant bootstrap value with known reference sequences. The three isolates clustering with subtype G had also been found to be subtype G in *gag* and 99CMLB33 was found to be F2 in *gag* and *pol*.

An interesting finding among the *pol* sequences was the identification of the subtype J sequence in the protease region of the 99CMLB40 isolate (see section 3.1.3.2). Very recent studies have also reported protease subtype J among subtype A isolates from the central part of Cameroon (Tscherning-Casper *et al*., 2000a; Fonjungo *et al*., 2000)

#### 3.1.3.1.3 Env sequences

Most genotypic studies of HIV have focused on the env gene since it is the most variable compared to other genes, and also contains the V3 loop, which is important in viral pathogenesis and tropism. Initial classification of HIV-1 was based on partial env sequences particularly the C2-V5 region (Myers *et al*., 1992). Fig. 3.3C shows a neighbour-joining tree of the V3-V5 region of the envelope gp120 region of the 19 primary isolates in relation to reference HIV-1 subtypes. The majority of the new isolates clustered among subtype A (14 of 19) with more than half of them being similar to CRF02.AG-like viruses (10 of 14) including the isolate 99CMLB20 which was unclassifiable in *pol*. The $A^{gag}$, $J^{pol}$ isolate (99CMLB40) was unclassifiable in env. The three subtype $G^{gag/pol}$ isolates and the subtype $F2^{gag/pol}$ isolate were of the same subtype in *env*.

Taken together the following forms of HIV-1 were identified in the panel of isolates originating mainly from Western Cameroon: $A^{gag}/A^{pol}/A^{env}$ (n=4); $G^{gag}/G^{pol}/G^{env}$ (n=3); $F2^{gag}/F2^{pol}/F2^{env}$ (n=1); $A^{gag}/AG^{pol}/AG^{env}$ (n=2); $AG^{gag}/A^{pol}/AG^{env}$ (n=1); $AG^{gag}/AG^{pol}/AG^{env}$ (n=6); $AG^{gag}/U^{pol}/AG^{env}$ (n=1) and a novel $A^{gag}/J^{pro/rt}/A^{int}/U^{env}$ complex recombinant (n=1). Table 3.1 gives a detailed overview of these results.

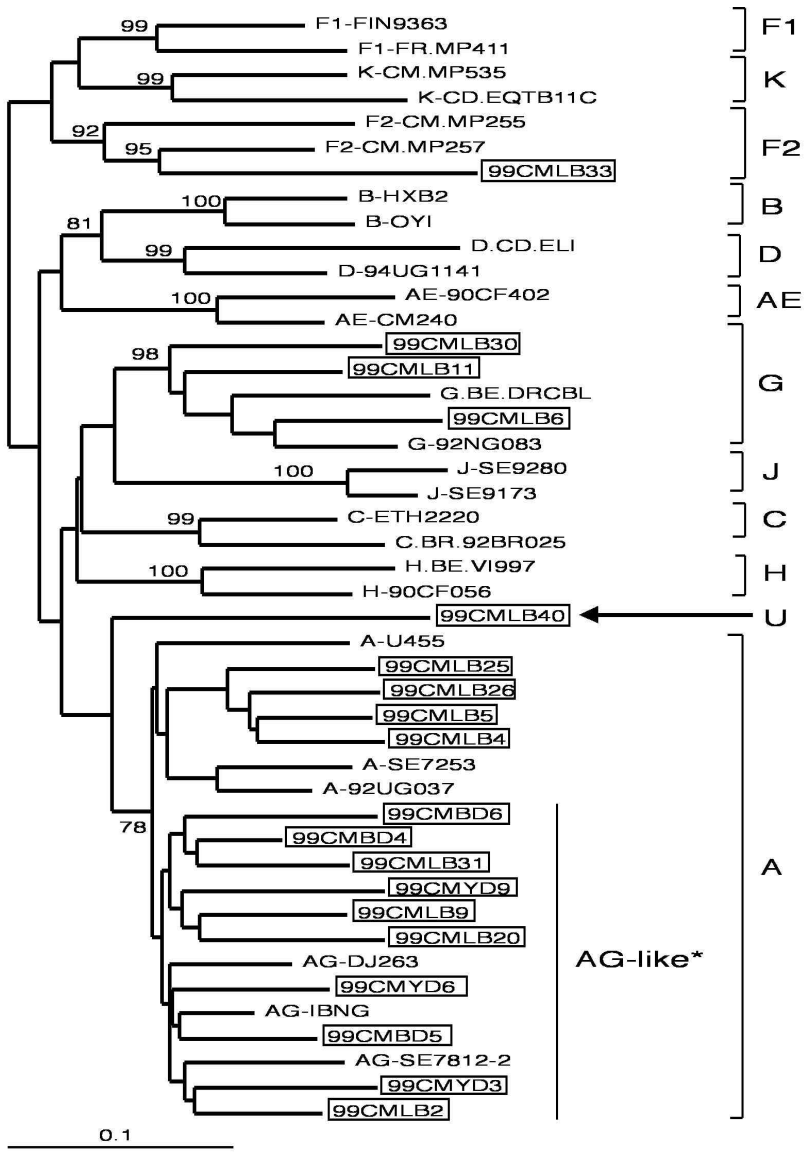


**Fig. 3.3C:** Env phylogenetic tree of the 19 HIV isolates from Wesern Cameroon in relation to reference HIV-1 strains. The unclassified 99CMLB40 sequence is shown by an arrow. Construction of the tree was the same as described for fig. 3.3A.

### 3.1.3.2. Evidence for a novel A/J/A mosaic *gag-pol* structure in 99CMLB40

Partial *gag, pol*, and *env* analyses of the isolate 99CMLB40 indicated it might be an A/J/U recombinant. In order to confirm this and precisely map the crossover points, the entire *gag-pol* region was amplified as a single fragment using long PCR, cloned into a plasmid and sequenced. Using the bootscanning method as implemented in the SIMPLOT program, the cross over sites were identified (fig. 3.4A). The query sequence 99CMLB40 was compared with the supposed parental subtypes A (92UG037.1) and J (SE9280-9). The entire *gag* region as well as the 3' part of

integrase sequences were shown to be subtype A, while the protease, reverse transcriptase and 5´ part of integrase was subtype J (fig. 3.4A and B). The A/J crossover point was located at the *gag/pol* boundary between nucleotides 1490 and 1510 (counting from the first nucleotide of gag) similar to the observation made when the full-length sequence of the isolate 99CMBD6 from this panel of isolates was

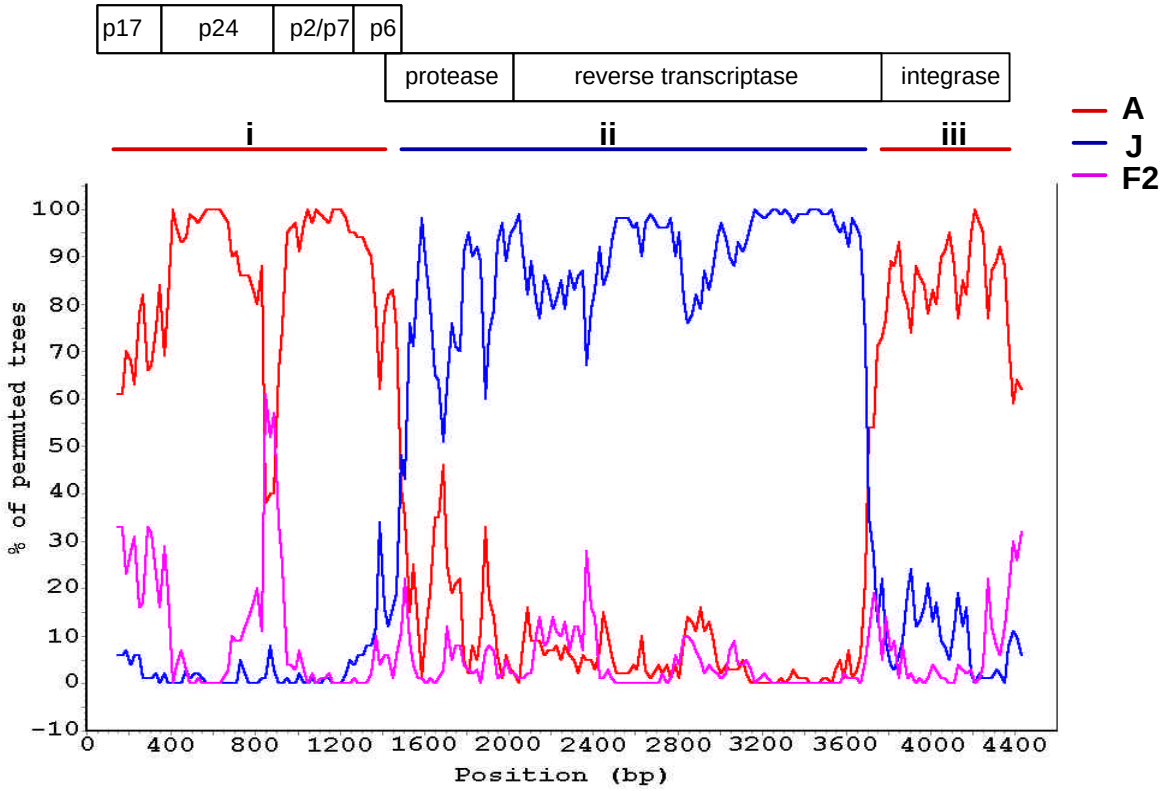


**Fig. 3.4 A**: Bootscanning analysis of *gag/pol* region of the novel AJU recombinant strain **99CMLB40.** In this analysis a window size of 300 bases with an increment of 20 bases was used. Regions with gaps were not included in the alignment. The Kimura 2-parameter method with 100 replicates was used. Sub-subtype F2 (95CMMP257C) was used as an out-group. The *y* -axis represents the bootstrap values while the *x* -axis shows the positions on the *gag-pol* alignment.

analysed (section 3.2.3.2) and what had been reported previously for other CRF02.AG (Cornelissen *et al*., 2000). The J/A crossover point lies within the 5' integrase sequence close to the boundary with RT between positions 3690 and 3710 also similar to the A/G cross over of 99CMBD6. The distinct A/J/A segments in the *gag- pol* gene of 99CMLB40 were analysed phylogenetically in relation to other reference sequences (fig. 3.4B). In the subtype A portions in *gag* and *pol*, the novel AJ sequence was more closely related to pure subtype A isolates than to the other

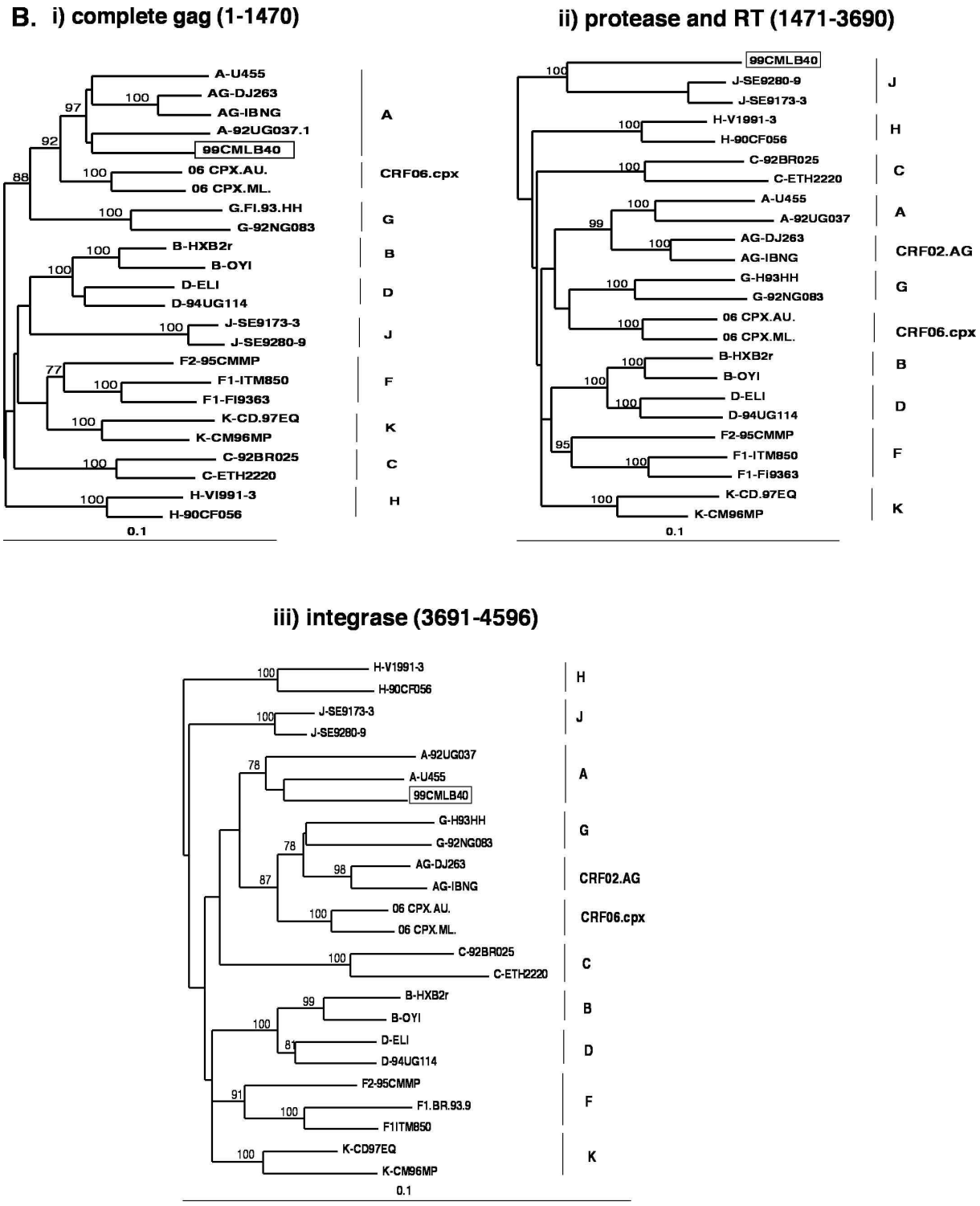


**Fig. 3.4B:** Phylogenetic relationship of i) complete *gag* ii) protease and RT and iii) integrase of the novel AJU complex recombinant strain (boxed). Trees were constructed after determining the crossover sites using the bootscanning method of the SIMPLOT program. The reference CRF06.cpx sequences are A/G/J strains from Burkina Faso and Mali.

recombinant A viruses (figs. 3.4B and 3.3A). The protease/reverse transcriptase region, on the other hand, clustered with the reference J sequences with a high bootstrap value (100 %; fig. 3.4B). Therefore, subtype A and J viruses are the

parental strains of isolate 99CMLB40. These findings were further confirmed after amplification and sequencing of regions spanning the crossover points from uncultured PBMC of this isolate. This supported the fact that this recombinant virus was already present in the patient and was not an artifact of *in vitro* cultivation.

### 3.1.3.3. Determination of putative drug resistance signature sequences in protease and reverse transcriptase

For any future drug application to be successful, knowledge about the prevalence of putative drug resistance signature sequences which might affect drug susceptibility is required. Studies of this type have been widely carried out using subtype B strains, which happen to be the most prevalent in the regions where anti retroviral medications targeting protease and reverse transcriptase are most available and commonly used (Condra *et al*., 1995, Molla *et al*., 1996). Only very few studies have been done to determine the prevalence of these signature sequences among drug naïve HIV infected Africans (Vergne *et al*., 2000).

Using the same *pol* sequences as in the phylogenetic analysis, the occurrence of variants corresponding to drug resistance polymorhism in subtype B isolates was determined using the Anti-viral Drug Resistance Analysis tool (ADRA). Analysis of codons 9-99 of protease did not reveal any of the known major polymorphims (D30N, G48V, I50V, V82A/T/F, I84V, or L90M) in any of the isolates. Polymorphisms were detected in 7 positions, namely L10V, G16E, K20R, M36I, D60E, L63P and V82I, which were previously shown to contribute to drug resistance in subtype B strains (fig. 3.5). Apart from the M36I polymorphism which was observed in all isolates the frequency of other alterations in the panel of isolates was generally low. Five, six and eight isolates respectively carried 3, 2 and 1 alterations in relevant positions. Fig. 3.5 shows these signature sequences in an amino acid alignment of the protease gene of all the primary isolates with the subtype B consensus sequence. When nucleotides 1-130 of reverse transcriptase were analysed only 2 alterations (V106I, V108I) were observed. In all, eight isolates did not carry any putative drug resistance polymorphism either in the protease or RT sequences analysed and there was also no subtype specific polymorphism observed.

```
             10         20         30         40         50         60         70         80         90       99
             *     *    *   *      * **  *          ***  *   *      *  *        *     *     * *   *  *
Consensus B  LVTIKIGGQL KEALLDTGAD DTVLEEMNLP GRWKPKMIGG IGGFIKVRQY DQILIEICGH KAIGTVLVGP TPVNIIGRNL LTQIGCTLNF

99CMBD4(AG)  V--V-----P I--------- ------I--- -K-------- ---------- ---------- ---------- ---------- ----------
99CMBD5(AG)  ---VR-D--- I--------- -----DI--- -K-------- ---------- ---------K ---------- ---------M ----------
99CMBD6(AG)  ---V-L---- I--------- ------ID-- -K-------- ---------- ---------K --V------- ---------M ----------
99CMLB2(AG)  ---VR-E--- I--------- -S----I--- -K-------- ---------- ---------K ---------- ---------M ----------
99CMLB31(AG) ---VR-E--P I--------- ------I--- -K-R------ ---------- ---H-----K --V------- ---------M ----------
99CMLB9(AG)  ---V------ R--------- ------I--- -K-------- ---------- ---P-----K ---------- ---------M ----------
99CMYD3(AG)  ---V------ I--------- ------I-F- -K-------- ---------- ---------K ---------- ---------M ----------
99CMYD9(AG)  --PV--E--- I--------- ------I--- -K-------- ---------- ---------K R--------- ---------M ----------
99CMLB4(A)   ---------- ---------- -----NID-- -K-------- ---------- ---------K ---------- ---------M ----------
99CMLB5(A)   ---V-----P I--------- ------I--- -K-------- ---------- ---------K R--------- ---------M ----------
99CMLB20(U)  ---VR----- I--------- ------I--- -K-------- ---------- ---------K ---------- ---------M ----------
99CMLB25(A)  V--V------ R--------- -----DI--- -K-------- ---------- ---------K ---------- ---------M ----------
99CMLB26(A)  ---V------ ---------- -----NID-- -K-------- ---------- ---------K ---------- ---------M ----------
99CMYD6(A)   V--V-----P I--------- ------I--- -K-------- ---------- ---------K ---------- ---------M ----------
99CMLB6(G)   ---V------ I--------- ------I--- -K-------- ---------- -------S-K ---------- --I------M ----------
99CMLB11(G)  --KVRV---- I--------- -----DI--- -K-------- ---------- E--------K ---------- --I------M ----------
99CMLB30(G)  --KV-V---- I--------- -----QI--- -K-------- ---------- -------E-K ---------- ---------M ----------
99CMLB33(F2) -----VE--- R--------- -----DI--- -K-------- ---------- --VH-----Q ---------- ---------M ----------
99CMLB40(J)  -----VA--- ---------- ------ID-- ---------- ---------- EE-----E-K ---------- ---------M ----------

Drug         1     2    3,4            3,5                        6  4,5                 7,8
```

**Fig. 3.5:** Protease amino acid sequences from drug naive HIV infected Cameroonians aligned with HIV-1 subtype B consensus sequence. Positions which have been shown to contribute to protease inhibitor drug resistance in subtype B are indicated with astericks. Amino acid residues 24, 30, 32, 33, 46, 47, 48, 50, 54, 71, 84, 88, and 90 are conserved among the Cameroonian isolates. Subtypes are indicated in parenthesis. Polymorphism were noticed at positions 10, 16, 20, 36, 60, 63, and 82. Residues at these positions are indicated in bold. The first 9 amino acids are encoded in the sequence corresponding to the 5' primer and therefore not included in this alignment. Numbers at the bottom of the alignment represent the various protease inhibitors to which the shown polymorphism contributes to drug resistance in HIV-1-M subtype B isolates (1 = MK639, 2 = ABT378, 3 = Ritonavir, 4 = Indinavir, 5 = Nelfinavir, 6 = DMP450, 7 = A-77003, 8 = XM 323). The V106I and V108I mutations, respectively, known to contribute to HBY095; DMP226, and Loviridae resistance were found in the 5' fragment of RT (data not shown).

It is possible that some drug resistance signature sequences occurring in minor variants of an isolate might not have been determined since sequence analysis were done directly from PCR products and not from individual molecular clones. It is of importance to phenotypically determine whether putative drug resistance polymorphisms observed among subtype B strains are specific only for this subtype or cut across the entire spectrum of HIV subtypes.

### 3.1.4. Biological Characterisation of primary isolates

#### 3.1.4.1. Biological phenotype, co-receptor usage and V3 loop sequences

The biological phenotype of the primary isolates were determined by infecting MT-2 cells and monitoring for syncytia formation. Six of the 19 isolates were syncytia inducing (SI) while 13 were non-syncytia inducing (NSI). The biological phenotype of the primary isolates on MT-2 cells could be predicted from the patient´s clinical status (table 3.1). Most SI isolates (5 of 6) were obtained from patients at late stages of AIDS while the NSI isolates were all from asymptomatic individuals or patients with AIDS related complex with the exception of 99CMLB40. Generally, *in vitro* characteristics such as biological phenotype and co-receptor usage of HIV strains have been widely used as markers of disease progression.

The biological phenotypes also correlated with co-receptor usage. All isolates were tested for their ability to infect GHOST cells expressing one of the following chemokine receptors: CCR1, CCR2, CCR3, CXCR4, CCR5, V28, BONZO, BOB (table 3.1). Irrespective of their subtype (genotype), all NSI isolates used CCR5 as their main co-receptor while all SI isolates used CXCR4. Three isolates used another co-receptor in addition to either CCR5 or CXCR4 and one SI isolate was found to be multi-tropic using CCR5, CXCR4, CCR3 and CCR1. None of the 19 isolates used CCR2, V28, BONZO or BOB for entry.

The V3 loop of HIV *env* has been shown to be important for antibody neutralisation, phenotypic changes and co-receptor usage (Cocchi *et al*., 1996). The V3 loops of 14 new isolates consisted of 35 amino acids, while two of either 34 or 37, and one of 33 amino acids were observed, respectively (fig. 3.6). The most common hexapeptide sequences at the apex of the V3 loop were GPGQAF and GPGQTF (7 and 6 of 19, respectively), while one isolate each contained the sequence GPGRAV, GAGQTF,

| Seq. ID | V3-Loop (1 11 25 35) | Net Charge | Ph. | Co-rec. usage |
|---|---|---|---|---|
| **Consens.A** | CTRPNNNTRK**S**VRI..***GPGQ***AFYATG**D**IIGDIRQAHC | +4 | NSI | R5 |
| **99CMBD4** | --------------..**----**T-----EV--------- | +4 | NSI | R5 |
| **99CMLB2** | ----G---------..**----**T--------------- | +4 | NSI | R5 |
| **99CMLB4** | -II--------IH-..**---------**R---------- | +4 | NSI | R5 |
| **99CMLB5** | -I-------RGIH-..**---------**R-A--------Y- | +4 | NSI | R5 |
| **99CMLB6** | -----------ITL..**----------**A---N------ | +5 | NSI | R5 |
| **99CMLB9** | ----------R-P-..**----**T----.----N------ | +5 | NSI | R5 |
| **99CMLB11** | -----------IS-..**----------------**KK-Y- | +3 | NSI | R5 |
| **99CMLB20** | -I-----AI-N-G-..**----**T--T--K---N-S--Y- | +3 | NSI | R5 |
| **99CMLB25** | -I--G------IH-..**---------**R---------- | +5 | NSI | R5 |
| **99CMLB26** | -I--------GIH-..**---------**R-----N--H-F- | +6 | NSI | R5 |
| **99CMLB31** | ---------T----..**----**T-----A------K-Y- | +4 | NSI | R5 |
| **99CMLB40** | ------Y----IP-..**---*R***-VFK----------Y- | +4 | NSI | R5,R3 |
| **99CMYD6** | ----H----T-T--..**----**T--------------- | +3 | NSI | R5 |
| **99CMBD5** | ----G------I--GI**-*R*--**T---N-RVV-------- | +7 | SI | X4,R1 |
| **99CMBD6** | ----G-PI--RIG-..**------**H---N------R-Q- | +6 | SI | X4 |
| **99CMLB30** | --------K-GI-TGI**-*A*--**T-----K---------- | +7 | SI | X4,R1 |
| **99CMLB33** | ----G-K--R-M--..**---*R***V----.N------K-Y- | +7 | SI | X4 |
| **99CMYD3** | ----G----RRM--..**---*Y***.--.-KR---------- | +8 | SI | X4,R5,R3,R1 |
| **99CMYD9** | ----A----RAIG-..**---*R***KY---DK---N------ | +8 | SI | X4 |

predicts CCR5 usage: **S/G**XXX..***GPGX***XXXXXX**E/D**

**Fig. 3.6:** Relationship between V3 loop amino acid sequences, biological phenotype and co-receptor usage of Cameroonian HIV-1 primary isolates. Seq.I.D = sequence identity; (.) = deletion in the amino acid sequence, (-) = amino acid identity; Ph = biological phenotype.

GPGRGF, GPGY.F, GRGQTF, GPGRKY. Apart from the GPGY and GAGQ motifs all others had been reported from Cameroon (Takehisa *et al*., 1998; Nkengasong *et al*., 1994; Mboudjeka *et al*., 1999b). A greater V3 loop sequence heterogeneity was observed among SI isolates.

The net amino acid charge of the V3 loop was previously found to be a determinant of biological phenotype (Chesebro *et al*., 1996; Xiao *et al*., 1998). All the NSI isolates except for one had a net charge of less than +6, while all SI isolate had a net charge of +6 or more (fig. 3.6). Xiao *et al*. (1998) determined a consensus sequence (S/GXXXGPGXXXXXXXE/D) proposed to be a predictor of CCR5 co-receptor usage. However, only 7 of the 13 NSI isolates contained this consensus sequence. Furthermore, uncharged residues (mostly serine / glycine) and negatively charged amino acids (mostly glutamic / aspartic acid) at positions 11 and 25 have been determined previously as predictors of the NSI phenotype (CCR5 usage) and positively charged amino acids in these positions as predictors of the SI phenotype (De Jong *et al*., 1992; Xiao *et al*., 1998) Accordingly, the majority of the NSI / CCR5

tropic isolates contained neutral or negatively charged residues in these positions, while 5 of the 6 SI/CXCR4 isolates contained positively charged residues at one of these positions. The SI subtype F isolate (99CMLB33) lacked positive amino acid residue at either position 11 or 25 in line with an observation made with subtype F viruses from Romania (Holm-Hansen *et al*., 1995). However, positive charges in position 11 or 25 were also observed in the case of 3 NSI isolates (fig. 3.6).

### 3.2. Characterisation of full-length infectious molecular clones of HIV-1

To investigate the genomic organisation and phylogenetic relationship, and to generate reference reagents that could be useful in vaccine development, full-length infectious molecular clones of HIV-1 are necessary. Two full-length infectious molecular clones were generated, one, CMH2.3 representing the most variable HIV-1 group O, predominant in Central Africa and the other, BD6.15 representing CRF02.AG, the most dominant circulating recombinant form of HIV-1 in Africa.

#### 3.2.1. Strategy used to derive full-length clones

Long range PCR procedures fail to generate full-length molecular clones of HIV-1 because of the sequence redundancies of the LTRs. The entire HIV genome was therefore amplified in two halves as shown schematically in fig. 3.7. The technique combines long PCR (Barnes, 1994) and provirus assembly of two amplimers (Herchenröder *et al*., 1995).

For the HIV-1-O molecular clone, four independent PCR 5’ fragments spanning the 5’LTR to *vpr* (5.7 kb) were generated and cloned into a vector. The 3’ fragments were amplified from the region spanning the *vpr* to the 3’LTR (4.2 kb) and directly subcloned into the plasmid containing the 5’ fragment using an Mlu I site introduced through a PCR primer. This artificial restriction site does not alter the coding sequence or known *cis*-acting elements. Since HIV primary isolates are made up of a swarm of replication competent as well as defective genomes, it was necessary to generate several full-length molecular clones from each isolate to increase the chances of obtaining an infectious clone. Twenty five full-length clones were generated using this approach.

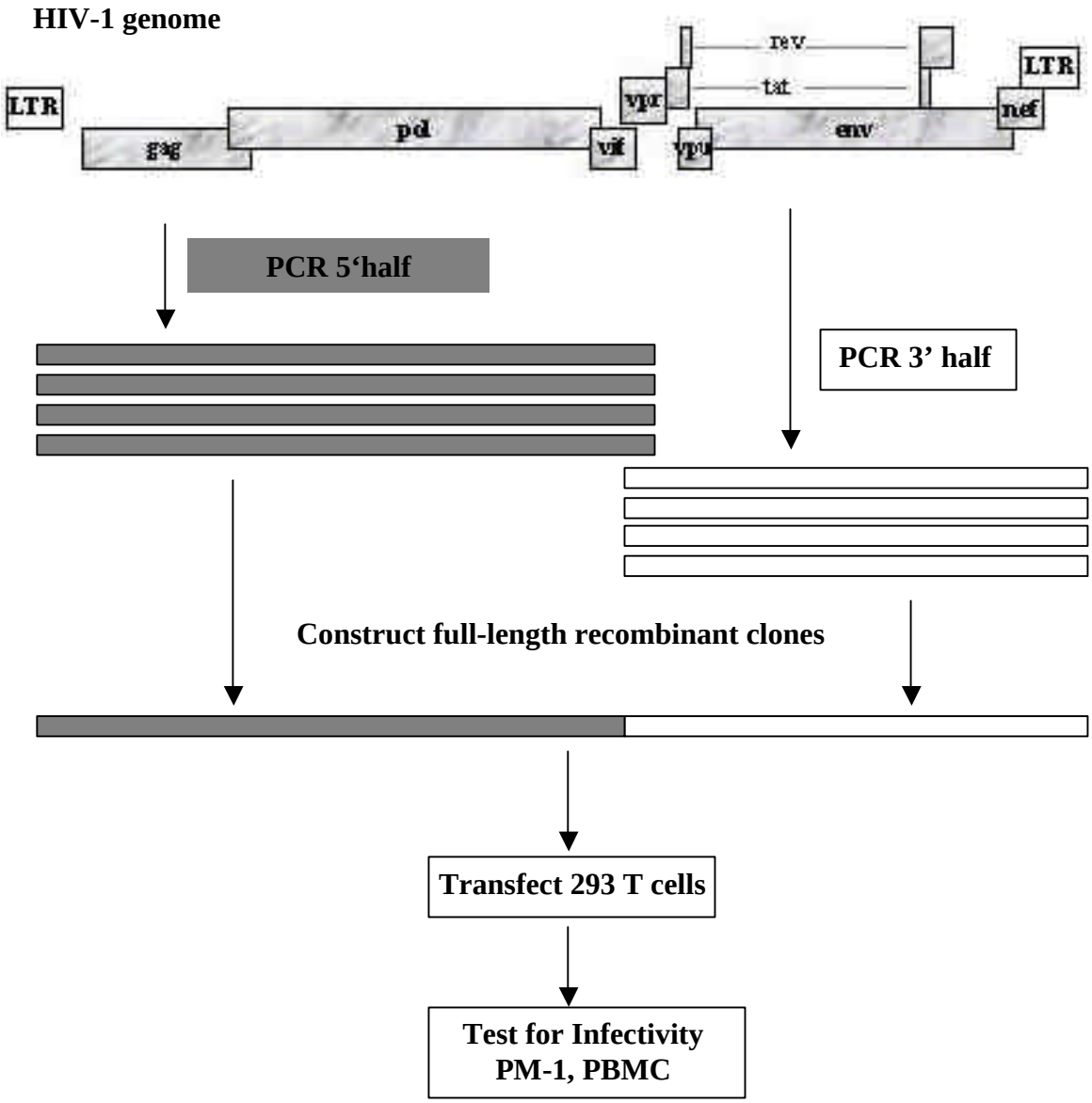


**Fig. 3.7:** Strategy used to generate full-length infectious molecular clones of HIV-1. The entire genome was amplified in two halves; from the beginning of the 5'LTR to the RT or *vpr* and then to the 3'LTR. The "chessboard" strategy was then used to generate full-length clones whose infectivity was tested by transfecting 293T cells and then later infecting different cell types with filtered transfection supernatant.

For the CRF02.AG clone, the 5' fragment covered the region between 5'LTR and the RT (2.7 kb) while the 3' fragment (7 kb) stretched from the RT to the 3'LTR. Both fragments were first introduced separately in the vector pCR-XL-TOPO utilizing the TA cloning method. Completion of the clones was done through an artificial Sal I site in RT introduced via the PCR primer.

### 3.2.2. Biological and molecular characteristics of HIV-1-O clone CMH2.3

The source of the DNA was an HIV-1 group O isolate, MVP8913 obtained from a 21 year old asymptomatic HIV-1 positive female Cameroonian in 1995 (Dittmar *et al*., 1999). Twenty-five full-length clones were generated and tested for infectivity by transfecting 293T cells with these clones and then infecting primary cells (e.g. PBMCs) or T-cell lines (e.g. PM-1) with transfection supernatants. Although all the 25 clones produced p24 antigen after transfection, only three produced virus that could infect either PBMCs or PM-1 cells. Two of these transfection derieved viruses infected different cell lines to levels about ten-fold less than the parental isolate.

| **Virus** | **V3 Loop sequence** | **NC** | **Cell type** | | | | | |
|---|---|---|---|---|---|---|---|---|
| **HIV-1-O** | | | **PBL** | **C8166** | **PM1** | **MT2** | **X4** | **R5** |
| ANT70 | CERPQI.DIQEMRIGPMAWYSMGIGGTA..GNSSRAAYC | 0 | + | (-) | (+) | NSI | - | + |
| 8913 | CERPWNQTVQEIRIGPMAWYSMGIIREEKSSNLSRLAYC | +1 | + | - | + | NSI | - | + |
| CMH2.3 | CERPWNQTVQEIRIGPMAWYSMGIIREEKSSNLSRLAYC | +1 | + | - | + | NSI | - | + |
| **HIV-1-M** | | | | | | | | |
| NL4-3 | CTRPNNNTRKSIRIQRGPGRAFVTIGKI:GNMRQAHC | +9 | + | + | + | SI | + | - |
| BD6 | CTRPGNPIRKR<u>I</u>GI..GPGQAFHATGNIIGDIRRAQC | +6 | + | + | + | SI | + | - |
| BD6.15 | CTRPGNPIRKR<u>V</u>GI..GPGQAFHATGNIIGDIRRAQC | +6 | + | + | + | SI | + | - |

**Table 3.2**: Biological characteristics of the two infectious molecular clones CMH2.3 and BD6.15 compared to their respective parental isolates in relation to their various V3 loops. Various T-cell lines in addition to primary PBLs were used for the infection experiments. For HIV-1-O, the prototype ANT70 was used as reference and its biological characteristic were adapted from the literature. NL4-3 is the laboratory adapted HIV-1-M subtype B strain. NC = net charge, X4 and R5 are GHOST cells respectively expressing the chemokine receptors CXCR4 and CCR5. The underlined amino acid residue indicates the difference between BD6 and BD6.15 in the V3 loop.

However, the third clone (designated CMH2.3) gave rise to virus which replicated in various cell lines to levels comparable to the parental isolate. Table 3.2 outlines the various biological characteristics of this clone compared to its parental isolate and another reference HIV-1-O strain, the ANT70 isolate (De Leys *et al*., 1990, Vanden Haesevelde *et al*., 1994). Infection experiments with PBMCs showed that viruses derived from transfections with the CMH 2.3 clone, infected primary cells and replicated with similar kinetics as the parental primary isolate. However, virus titers

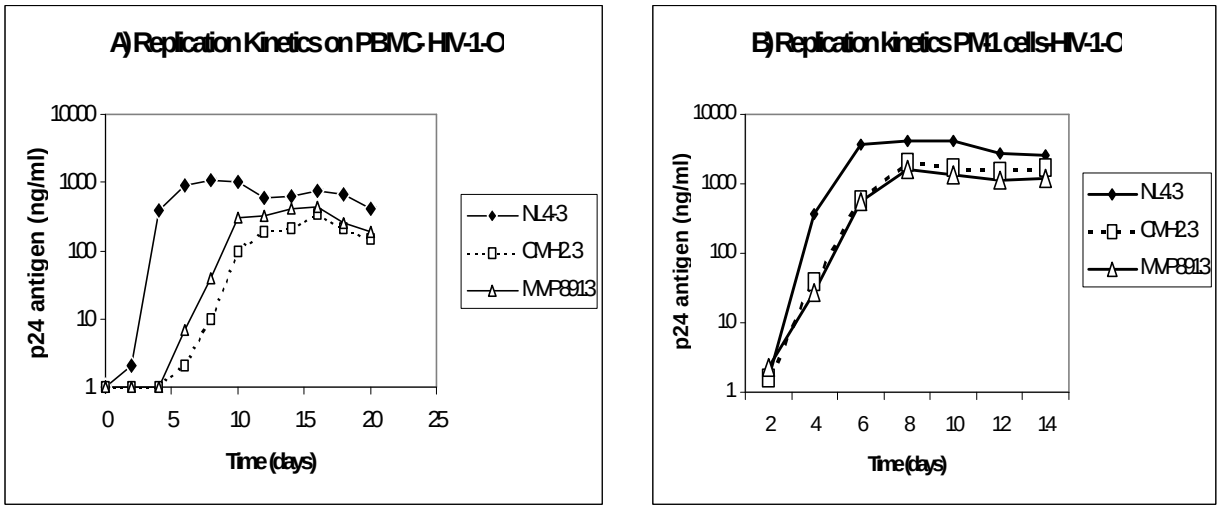


**Figure 3.8**: Replication kinetics of the infectious molecular clone CMH2.3 representing HIV-1-O in A) PBMC and B) PM-1. Filtered transfection supernatant was used to generate virus stocks and afterwards 1000$TCID_{50}$ and 500$TCID_{50}$ of this was used to respectively infect both cell types. Viral growth was monitored by sampling for p24 antigen after every 2 days. Experiments were done in quadruplicate.

were about 2 fold lower for transfection derived virus compared to the parental virus (fig. 3.8A). Replication kinetics in PM-1 cells, on the other hand, were completely identical for both viruses reaching peak p24 production on day 8 post infection (fig. 3.8B).

The biological phenotype and co-receptor usage of the primary isolate and the infectious molecular clone were identical (table 3.2), i.e. they were both non-syncytium inducing viruses (NSI) and used CCR5 as co-receptor.

Electron micrographs of infected PM-1 cells confirmed the production of virus progeny by transfection derived virus as well as by the parental isolate (fig. 3.9A). Western blot analysis using polyclonal antisera specific to matrix, capsid, integrase, and RT of HIV-1-M subtype B, as well as pooled HIV-1-O patient sera indicated some differences between HIV-1-O and the HIV-1-M subtype B clone (NL4-3). The molecular size of the HIV-1-O capsid seem to be a bit larger than the respective 24 kD protein of HIV-1-M, an observation made previously by Gürtler et al., (1994). Also, the polyclonal antisera against matrix for group M (at least subtype B) was not able to

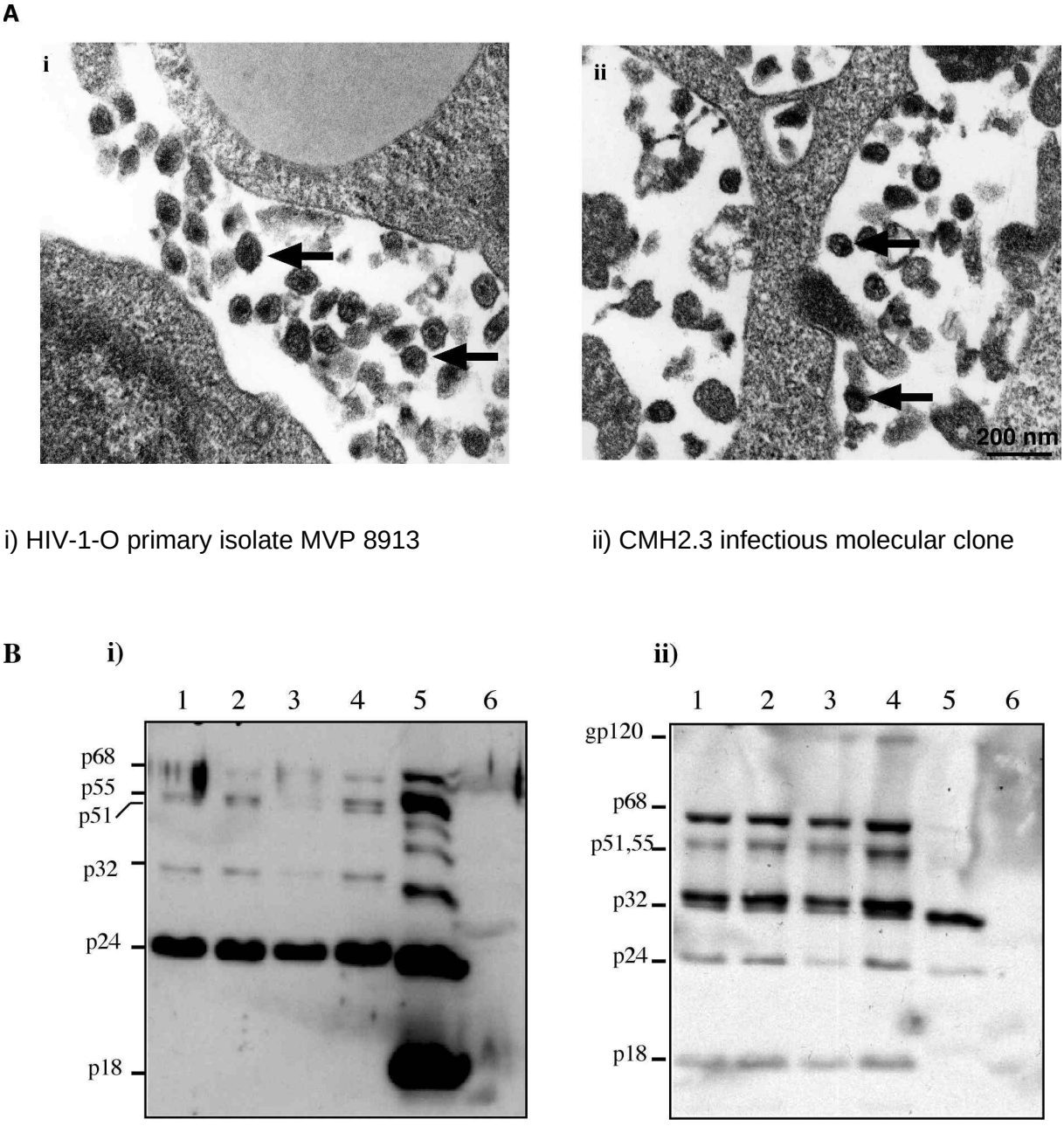


**Fig. 3.9 A)** Electron micrographs of virus particles released from PM-1 cells (arrows show examples of particles) infected with HIV-1-O. **i**) MVP8913 primary isolate and **ii**) CMH2.3 molecular clone. Cells were infected with filtered virus supernatant and cultivated for one week. These were later stained and observed under the electron microscope as described in materials and methods. **B)** Western blot of PM-1 cells infected with transfection supernatants of various molecuar clones. No.1 and 2 are HIV-1-O clones which replicated to levels significantly lower than the parental HIV-1-O isolate and were therefore not further characterised. No.4 is the infectious molecular clone CMH2.3 while No.3 is a second passage of CMH2.3. No.5 is NL4-3 and No.6 is the mock control. **i**) polyclonal antisera to MA, CA, RT and IN of HIV-1-M were used. The MA antisra did not react with the matrix of HIV-1-O. **ii**) A pool of HIV-1-O patient sera was used. This sera was relatively less ractive against viral proteins of NL4-3 (HIV-1-M).

**A.** MVP8913 primary isolate **B.** CMH2.3 molecular clone

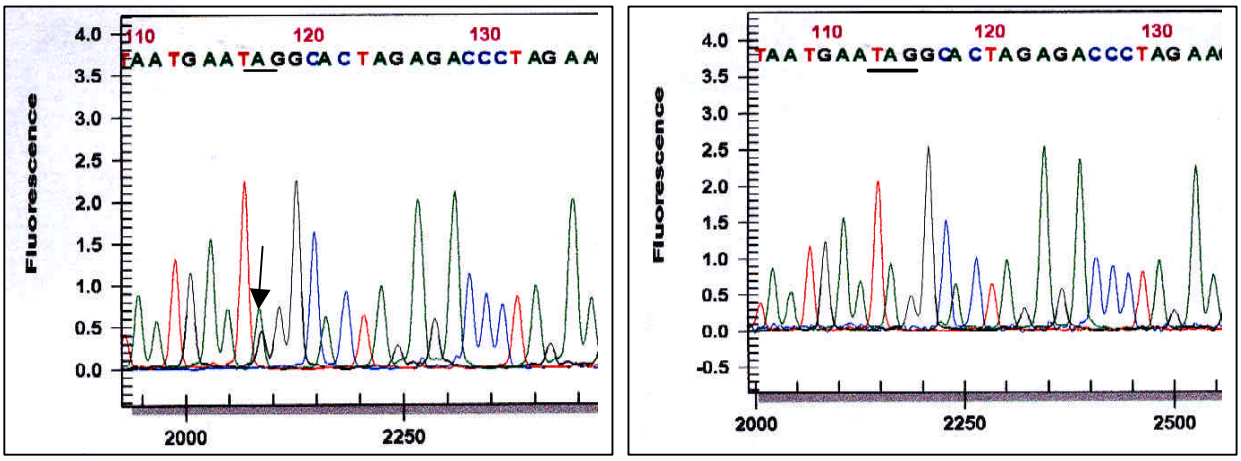


**Fig. 3.10**: Electropherogram showing the in-frame stop codon and the presence of two different variants (double peak and arrow) in the *vpr* of the HIV-1-O primary isolate MVP8913 (A) and a single population with a stop codon in the infectious molecular clone CMH2.3 (B). The nucleotide sequences are represented at the top. The nucleotides (TAG) coding for the in-frame stop codon are underlined in both A and B.

detect the HIV-1-O matrix protein. When pooled HIV-1-O patient antisera was used, the p18 of HIV-1-O and not HIV-1-M (at least subtype B), could be detected (fig. 3.9B). This lack of cross reactivity between HIV-1-O sera and HIV-1-M matrix protein was also observed when a commercial subtype B Western blot was used (fig. 3.1). Generally, cross-reactivity of the HIV-1-O sera towards viral proteins of NL4-3 (HIV-1-M) was low.

Complete sequencing of the CMH2.3 proviral genome showed that it was composed of 9868 base pairs. All the reading frames of the major structural proteins *gag*, *pol* and *env* were open while the accessory protein, *vpr* contained an in-frame stop codon. Direct sequencing of a PCR product of the amino terminal region of *vpr* from the primary isolate indicated the presence of two different populations; one containing and the other lacking this in-frame stop codon (fig. 3.10). When individual clones of this fragment were analysed, only one in five of the clones did not contain this stop codon, indicating the dominance of the variant containing this stop codon in the primary isolate. Searching the HIV database for the sequences of HIV-1 infectious molecular clones revealed that about 50 % of infectious molecular clones generated so far had at least one defective accessory gene, especially *vpr* or *vpu*. The defective *vpr* was due mainly to in-frame stop codons, while the defective *vpu* gene was as a result of the loss of a start codon (Gao *et al*., 1997).

Furthermore, the second exon of *tat* was prematurely terminated 17 amino acids before the usual stop found in other HIV-1-O isolates. This truncated tat protein was also observed recently for the 99SE-MP1299 group O strain (Kane *et al*., 2001). Gao *et al*., 1997 had previously described in-frame stop codons, 13 to 16 amino acids from the carboxyl terminal end of the second exon of tat in some subtype D viruses. This change does not appear to affect the function of Tat since its known functional domain does not lie in this region. The transcriptional factors such as the two NF-κB, and SP1 binding sites and the TATA-box motif of the LTR were all conserved. Analysis of the gag sequence revealed that the proline-rich motif P-XXXX-P-XX-P-XXXXX-P which is responsible for the incorporation of cyclophilin A was conserved. Cyclophilin A is a cellular protein that is packaged into viral particles by HIV-1 and not HIV-2 or SIV. It is necessary for the replication of HIV-1-M, some HIV-1-O but not HIV-2 or SIV (Braaten *et al*., 1996, Wiegers and Kräusslich, in press). The major homology region important in the virus assembly process was also highly conserved. Examining the RT showed that the amino acid mutations A98G, V179E and Y181C in relation to HIV-1-M which have been associated with the natural resistance of HIV-1-O isolates to non-nucleoside reverse transcriptase inhibitors (NNRTIs) were all conserved (Quinones-Mateu *et al*., 1997). In HIV-1 group O viruses the V3 region of the envelope gp120 is usually composed of 36 to 39 amino acids. The V3 region of both the CMH2.3 clone and its parental primary isolate were identical, and each was composed of 39 amino acids, similar to that of the MVP5180 prototype HIV-1-O isolate (Gürtler *et al*., 1994; table 3.2). However, examining the hexameric tip of this loop showed it was composed of the GPMAWY motif, identical to that of ANT70 (table 3.2). The envelope of CMH2.3 clone contained 32 predicted glycosylation sites, three more than found in ANT70 prototype strain. Env proteins are cotranslationally glycosylated in the rough ER by cellular enzymes that attach mannose-rich oligosacharides to the asparagine in Asn-X-Ser or Asn-X-Thr motifs. On average, HIV contains about 30 glycosylation sites in env, approximately 25 of these are located in gp120. The heavy glycosylation of gp120 contributes to the reduction of protein epitope exposure and enhances viral evasion from antibody (Back *et al*., 1994). Phylogenetic analysis showed that CMH2.3 clusters with HIV-1 group O isolates and is more closely related to ANT70 than to MVP5180 (fig. 3.11A). This was also confirmed when *gag, pol, env* and *nef* fragments were analysed individually.

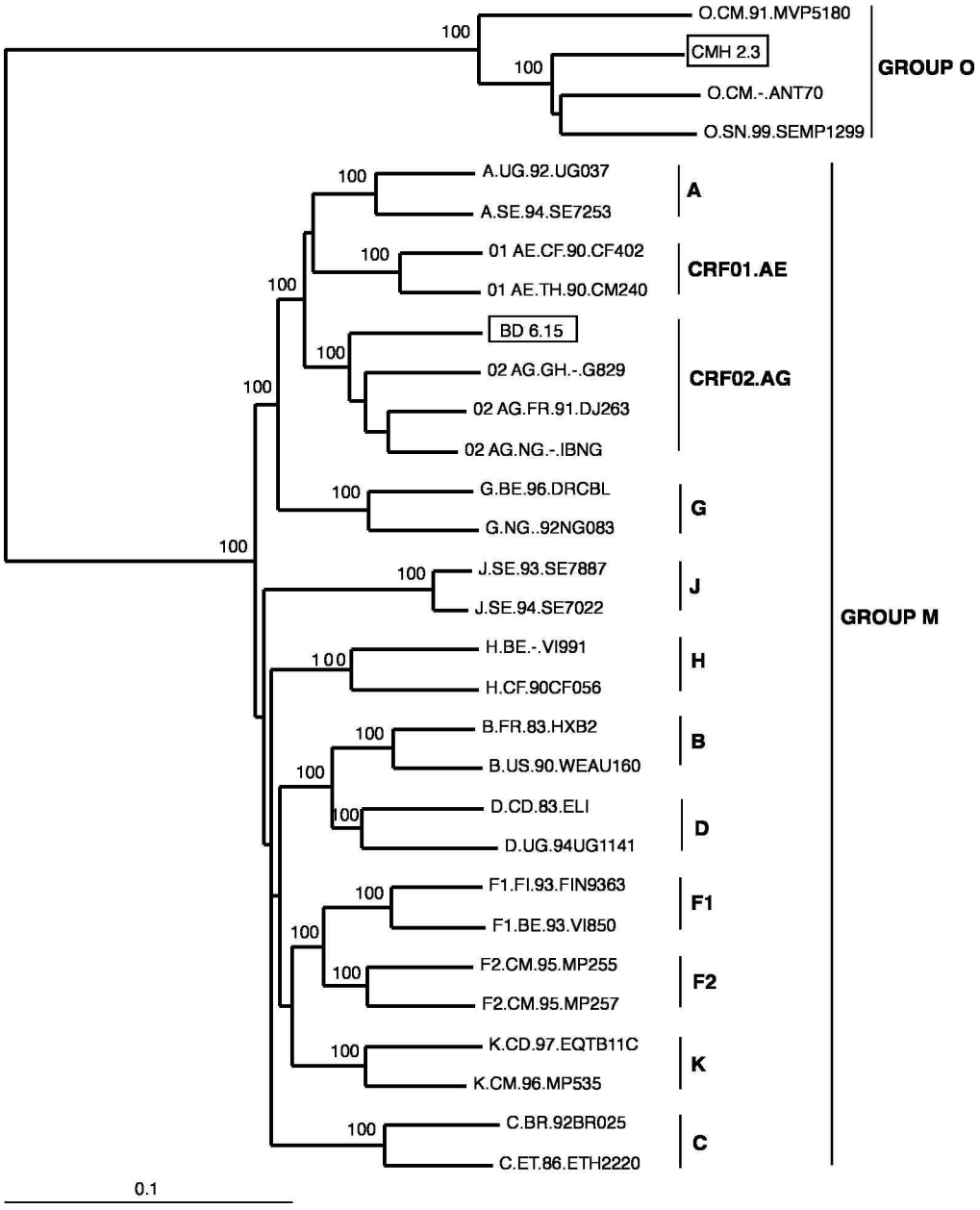


**Fig. 3.11A :** Phylogenetic relationship of full-length genomic sequences of the two infectious molecular clones (boxed) with other HIV-1 strains. The tree was constructed using the neighbour joining method and reliability of the branching pattern confirmed by bootstrapping (100 replicates). CMH2.3 clusters with HIV-1 group O while BD6.15 is a member of the CRF02.AG lineage mostly found in West and Central Africa. The HIV-1 subtypes and groups are represented to the right.

Fig. 3.11B shows the phylogenetic relationship of CMH2.3 with the full-length envelope sequences of HIV-1-O isolates available in the HIV database. Since partial genome analysis does not allow the detection of recombinants and considering the fact that inter-group recombinant between HIV-1-M and -O have been reported

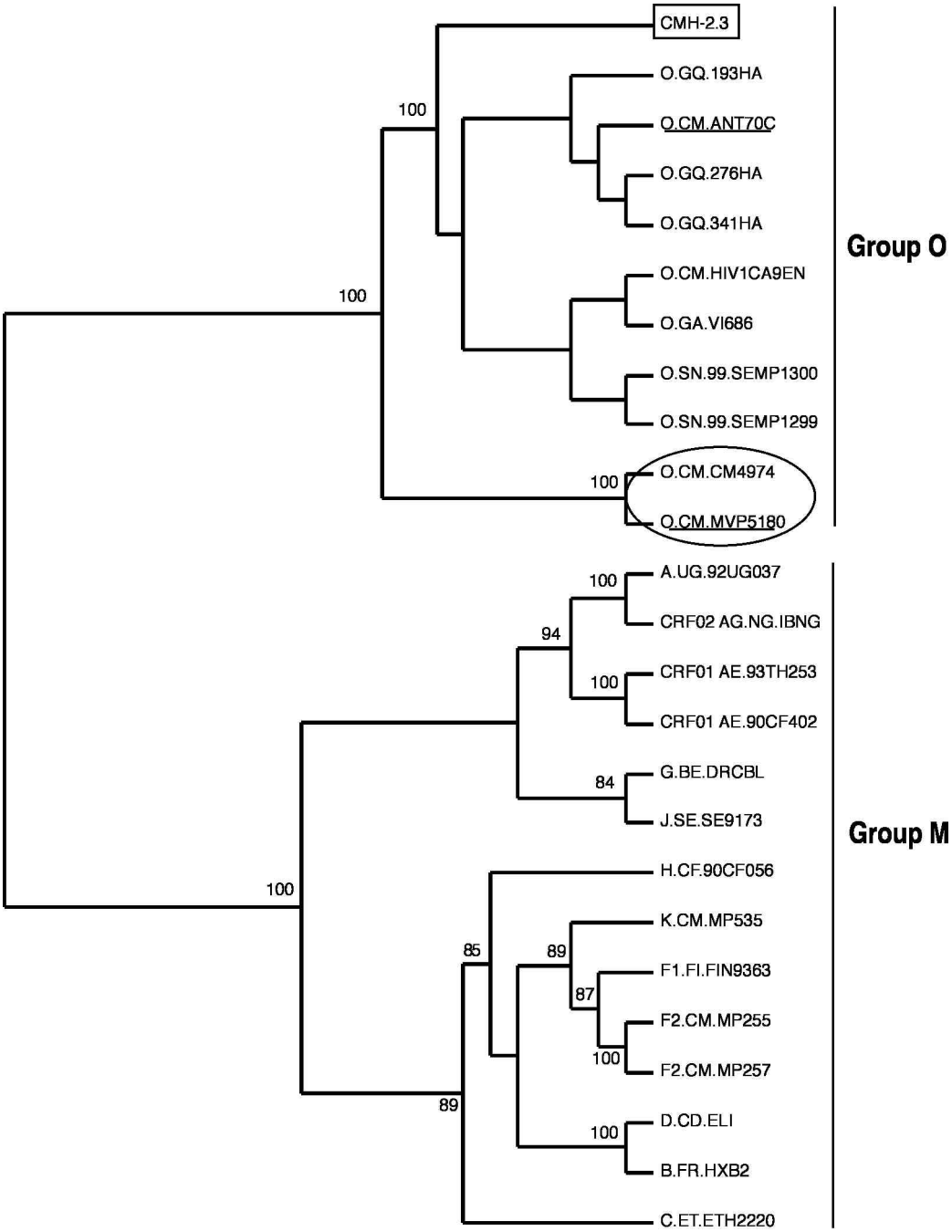


**Fig. 3.11B:** Phylogentic relationship of full-length envelope region of HIV-1-M and -O isolates. The subtypes for group M are found at the beginning of each isolate identification. Group O isolates are not divided into subtypes. The newly sequenced CMH 2.3 isolate is boxed while the underlined isolates are the prototype strains of HIV-1-O. Most HIV-1-O isolates are closer to the CM.ANT70 isolate than the CM.MVP5180 strain. Only the CM.CM4974 isolate clusters with CM.MVP5180 (circled). CM indicates isolate is from Cameroon.

(Takehisa *et al*., 1999), the entire genome of CMH2.3 was analysed using bootscanning and the similarity plot. These analysis confirmed that the CMH2.3 clone was indeed a non-recombinant HIV-1-O isolate. This clone therefore represents the first infectious molecular clone of HIV-1 group O to be generated and characterised.

### 3.2.3. Characteristics of the CRF02.AG infectious molecular clone BD6.15

#### 3.2.3.1. Biological characterisation

The primary isolate from which the BD6.15 clone was derived has been described in section 3.1. This isolate was selected as a representative of the CRF02.AG viruses which are the most prevalent type of HIV in West and Central Africa (Heyndrickx *et al*., 2000, Montavon *et al*., 2000). The "long PCR" approach was used to amplify the entire genome in two fragments as described in section 3.2.1. After obtaining respectively four clones each for the 5' and 3' fragments, a total of 16 full-length

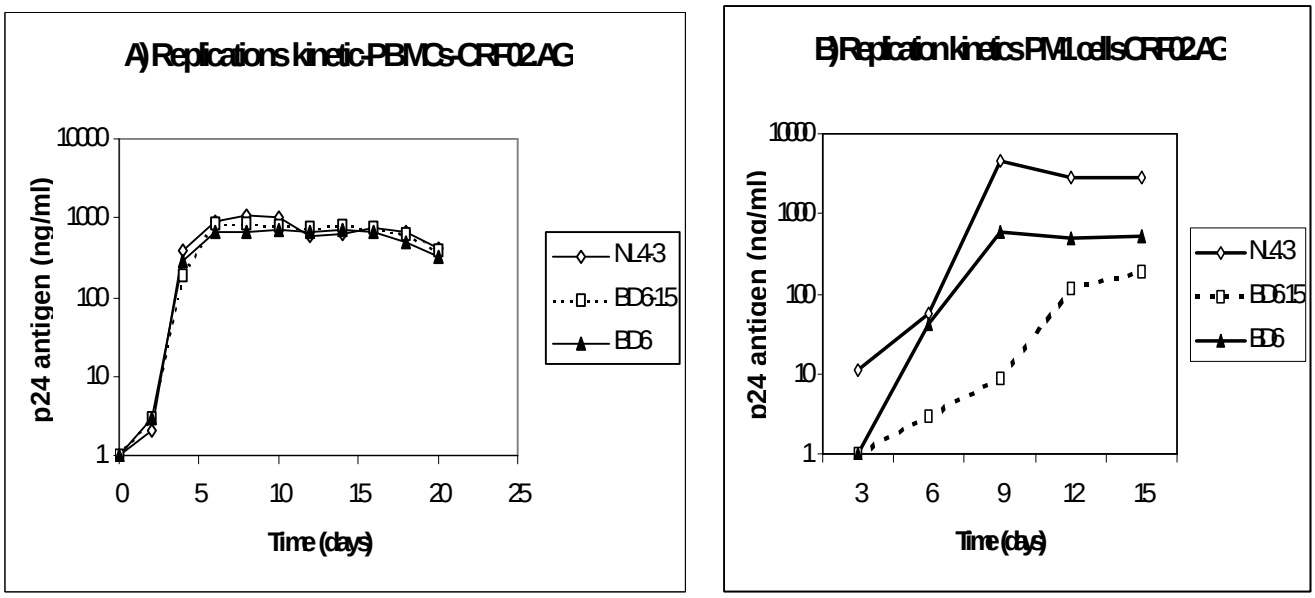


**Fig. 3.12:** Replication kinetics of the BD6.15 clone a member of the CRF02.AG group of viruses in **A)** PBMCs and **B)** PM-1 cells. Infection experiments were done as described for the HIV-1-O clone and p24 monitored after 2-3 days for 2 weeks (PM-1) and 3 weeks (PBMCs). The results shown are the mean of a single experiment done in quadruplicate. BD6 is the primary isolate from which the infectious molecular clone BD6.15 was obtained.

clones was then generated by using a "chessboard" cloning strategy (fig. 3.7). These clones were then tested for infectivity as described for the HIV-1-O infectious molecular clone in the previous section. After transfection of 293T cells, all 16 clones produced p24 antigen. Infection experiments with primary PBMCs indicated that only transfection derived virus from the clone designated BD6.15 could replicate in these cells to similar levels like its parental isolate (fig. 3.12A). This tranfection derived virus, its parental primary isolate and the NL4-3 virus all showed peak p24 production between days 8-10 post infection. The fact that BD6.15 was obtained from primary

PBMCs coupled with its similar replication kinetics in these cells makes it a good tool for studying the molecular characteristic of CRF02.AG viruses. In PM-1 cells, BD6.15 replicated to levels relatively lower than its parental isolate (fig. 3.12B). No possible explanation for this could be found at present and therefore requires further analysis. Other infection experiments later showed that virus produced from this clone grew best on C8166, another T-cell line. Typing for co-receptor showed a similar CXCR4 usage of both the BD6.15 clone and its parental isolate (table 3.2). The biological phenotype on MT-2 cells of both the clone and the isolate were syncytium inducing.
Sequencing of the entire BD6.15 provirus revealed that it is 9,774 base pairs long with the arrangement 5'LTR, *gag*, *pol*, *vif*, *vpr*, *tat*, *vpu*, *rev*, *env*, *nef* and 3'LTR. All the reading frames were open except *vpr* which had an in-frame stop codon at its 3' end. Like its parental isolate the V3 loop of BD6.15 was made up of 37 amino acids, identical to its parental isolate but for a single amino acid difference (table 3.2). The V3 loop contained the GPGQAF motif which is dominant among African HIV-1 isolates. The putative binding sites for transcriptional factors NF-κB, SP1 and TATA were all conserved. A 16 base pair insertion downstream of the primer binding site reported in some subtype A, G and almost all known CRF02.AG viruses was present. Some minor deletions and insertions were observed in the envelope region respectively at the carboxyl terminal of gp41 and gp120. In the amino terminal of Nef, an insertion of 13 amino acid residues was observed. Most interesting was the fact that 10 of these amino acids were a duplication of an amino acid sequence located just a few amino acids towards the amino terminus. Recently another CRF02.AG infectious molecular clone was described with an insertion at the same position, however the duplication was variable when compared to the BD6.15 clone (Kusagawa *et al*., 2001). This insertion which is not subtype specific, varies from 6 to 13 amino acids in different isolates. It remains to be seen whether these alterations have any consequences on the virological properties of this clone, especially its replication in PM-1 culture which was observed to be slightly lower than for its parental isolates.

### 3.2.3.2. Recombinant structure of BD 6.15 and other CRF02.AG viruses

To better understand the recombinant structure of BD6.15, the sequence of the entire genome was compared with other CRF02.AG full length sequences (fig. 3.11 A).

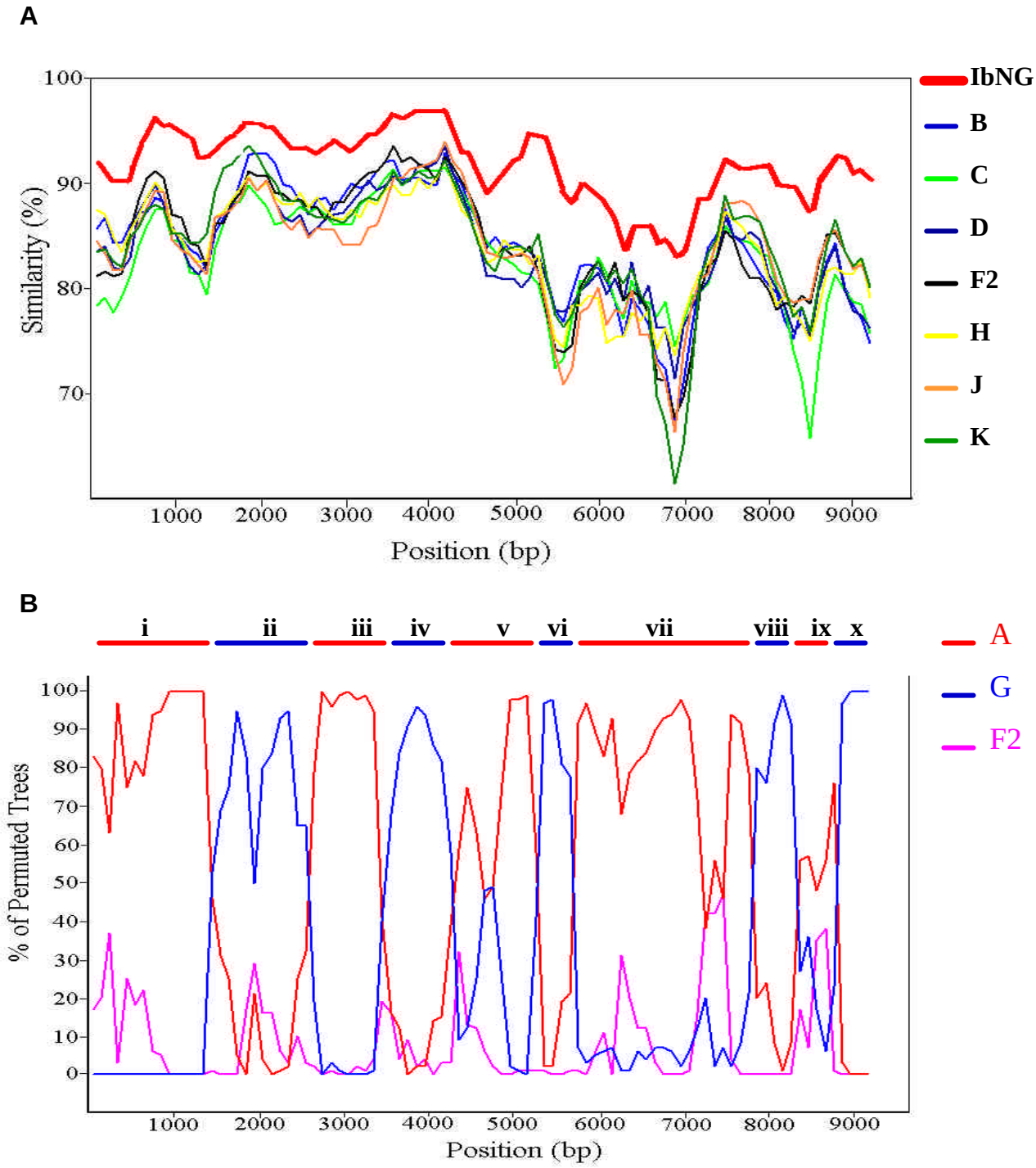


**Fig. 3.13** : **A)** Similarity plot of the full-length genome sequence of BD6.15 compared with reference sequences of the subtypes indicated on the right. In this analysis, a window size of 500 bases with an increment of 100 bases was used. BD6.15 is most similar to IbNG, the prototype strain of CRF02.AG viruses. **B)** Bootscan analysis of the BD6.15 sequence showing the crossover points relative to its putative parental subtypes A (red) and G (blue). Sub-subtype F2 was used as an out-group. The numbers at the bottom represent the positions on the HIV-1 genome counting from the start codon of *gag.*The roman numerals indicate the gene fragments used to construct the phylogenetic trees shown in fig. 3.13C.

Phylogenetic analysis of BD6.15 shows that it clusters with other members of the CRF02.AG lineage. Fig. 3.13A depicts a similarity plot in which the BD 6.15 isolate was compared to other subtypes including the CRF02.AG prototype strain IbNG. It clearly shows that this new sequence of BD6.15 is more closely related to the IbNg strain than to any other subtype. To determine the various recombination crossover points between putative parental isolates along the genome, the bootscanning analysis was used. This approach examines the genomic sequence in a given window size moving along in defined steps. This is represented in figure 3.13B and the phylogenetic trees supporting the various segments in fig. 3.13C. Nine of the ten fragments were confirmed to belong to either subtype A or G by a significant bootstrap value >70% in a phylogenetic analysis with reference sequences of all subtypes (fig. 3.13C). Fragment (ix) which stretches from nucleotide 7951-8450 was tentatively classified as subtype A due to its low bootstrap value of 52%. The CRF02.AG strain GH.G829 is unclassifiable in this region (fig. 3.13C(ix) and fig. 3.14). In fig. 3.14 the genomic structure of BD6.15 is compared with four other CRF02.AG recombinants: IbNg from Nigeria, CM53392 from Cameroon, 99GH.AG2 and GH.G829 from Ghana, and 99CMLB40, the AJU isolate from this study. The crossover points between subtypes A and G of BD6.15 were as follows (fig. 3.13B and C): nucleotide 1600 (A to G), 2550 (G to A), 3450 (A to G), 4050 (G to A), 5150 (A to G), 5650 (G to A), 7550 (A to G), 7950 (G to A), 8450 (A to G), Although the CRF02.AG recombinants shared most crossover points along their genomes there were some differences noticed between different strains. For example the region stretching from *vif* to *vpr* of the 99GH.AG2 isolate was unclassifiable but belonged to subtype A or G in the other CRF02.AG strains examined. Also, the *nef* gene of all the AG viruses were subtype G except for the two Ghanaian isolates 99GH.AG2 and GH.G829 which were respectively subtype A and unclassifiable. The subtype G portion at the 3' of *pol* in the CM53392 strain was the shortest among all the CRF02.AG isolates examined. Carr *et al*. (2001) also found out that although the CM533922 virus shared a similar recombinant structure with other CRF02.AG viruses, phylogenetically it clusters more closely to subtype A or G than to CRF02.AG viruses in each segment examined.

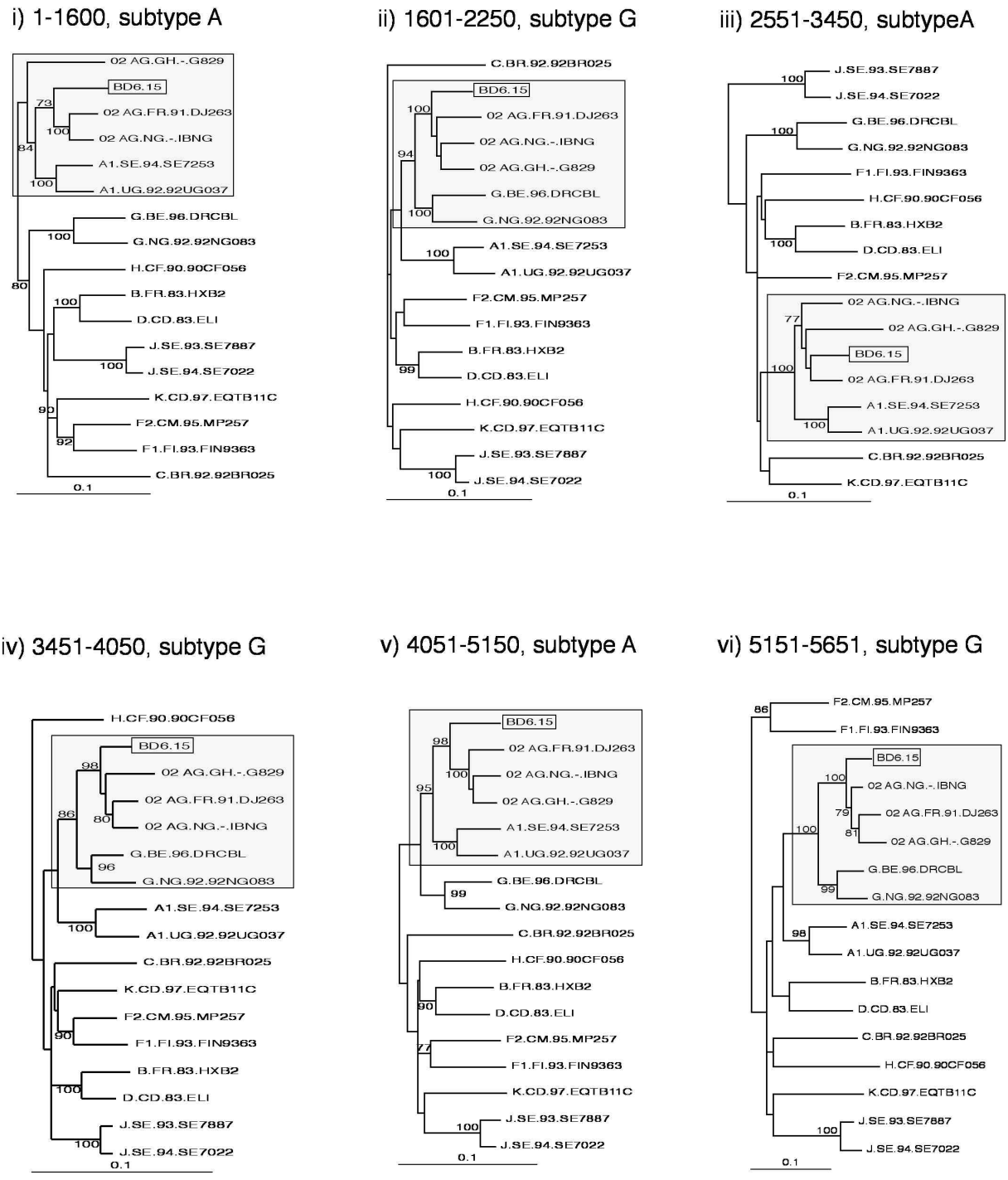


**Fig. 3.13C (i-x)**: See figure legend on next page.

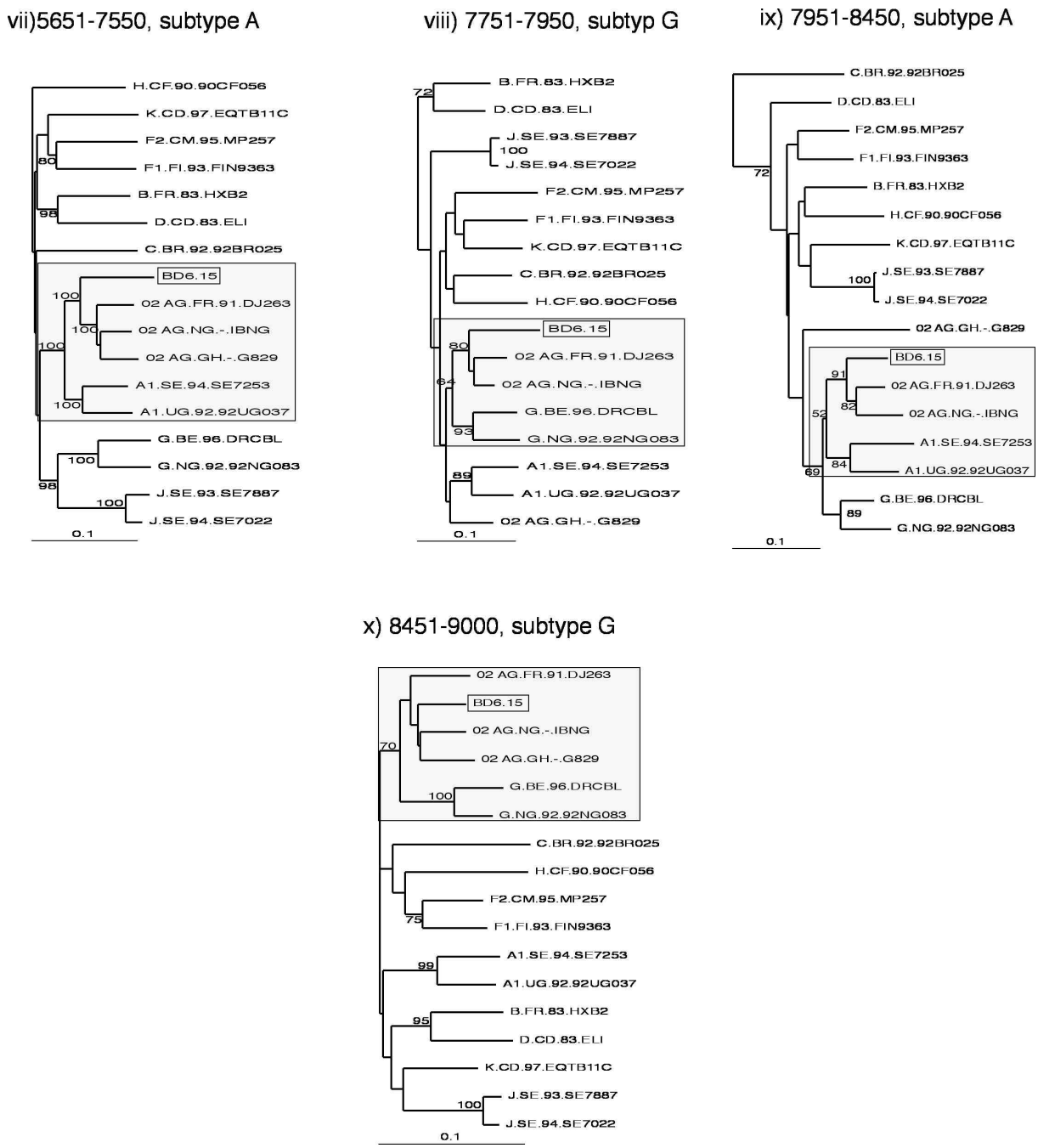


**Fig 3.13C (i-x, continued):** Neighbour joining trees depicting the discordant branching orders of the newly characterised BD6.15 virus from Cameroon in relation to other CRF.02AG viruses and HIV-1 reference strains. The trees were constructed following the crossover points between the putative parental subtpes A and G determined by means of the bootscanning analysis of the SIMPLOT program (see fig. 3.13B). Only bootstrap values above 70% were considered significant. The genome fragments used to construct each tree and the subtype of the BD6.15 virus is indicated at the top. The BD6.15 sequence is boxed while the subtype with which it clusters is shaded in grey.

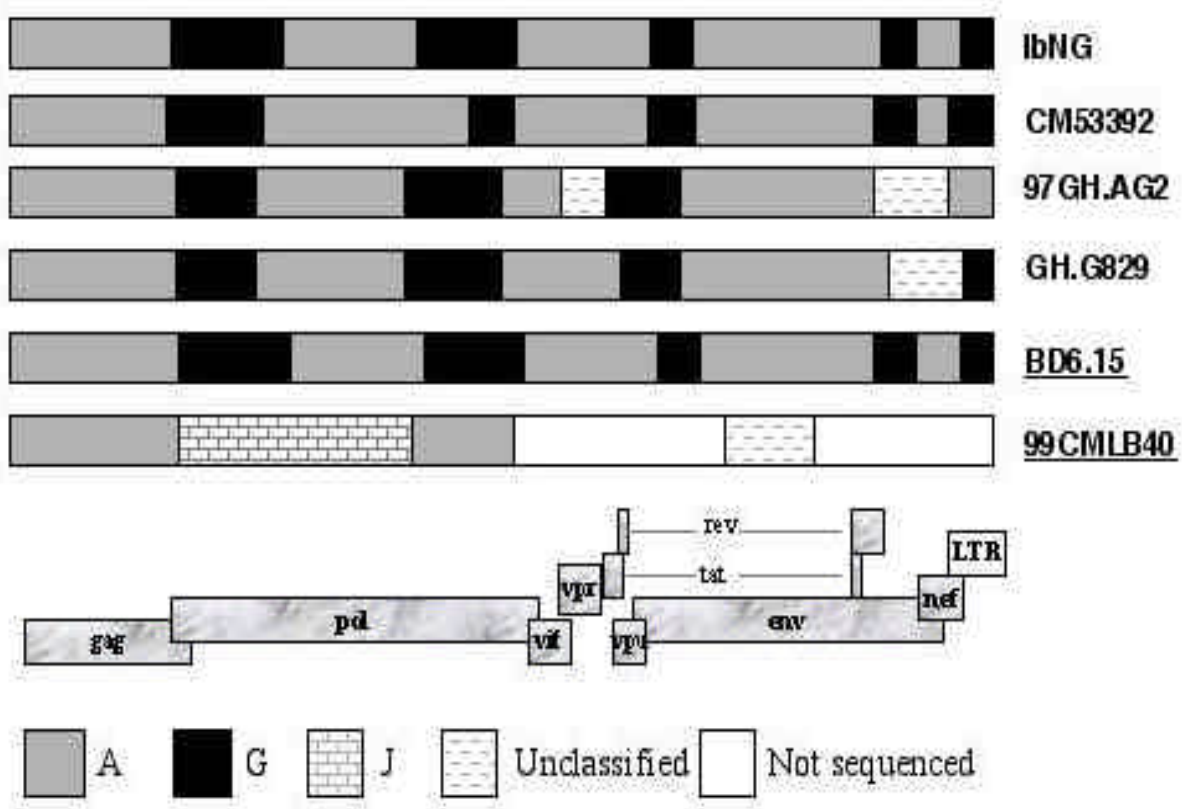


**Fig. 3.14:** Schematic representation of the recombination breakpoints of BD6.15 the CRF02.AG and 99CMLB40 the novel AJU recombinant viruses from Cameroon (underlined) analysed in this study. The other viruses were from the HIV database: IbNg is the prototype CRF02.AG strain from Nigeria, CM53392 is from Cameroon while GH.G829 and GH.G829 are from Ghana. A common feature shared by all these viruses is that their respective *gag* genes are made up of subtype A. The complete genomic structure of HIV-1 with the exception of the 5' LTR is shown at the bottom.

They suggested that AG recombinant viruses with nearly the same crossover points as CRF02.AG might have pre-existed before the epidemic spread of CRF02.AG viruses in West and West central Africa.

# 4. DISCUSSION

The present study provides a comprehensive analysis of HIV-1 strains circulating in Western Cameroon. This is the first study of its kind from Western Cameroon, a region located in the biogeographic transition zone between Central Cameroon and Nigeria, a neighbouring country to Cameroon. The study gives an insight into the biologic and genetic characteristics of the dominant HIV-1 strains circulating in this region. Briefly, the importance of this study relative to previous studies in Cameroon lies in the origin of the isolates and the depth of the study which allowed the generation of well characterised reference materials that will greatly facilitate the understanding of the functional relationship between HIV genotypes. In fact, the development of appropriate vaccines, comprehensive diagnostic assays and the tracking of drug resistant HIV-1 strains relies on understanding the molecular and biological characteristics of these strains in any given population. This is particularly critical in regions such as Cameroon where multiple subtypes, inter-subtype and inter-group recombinants of HIV co-circulate.

## 4.1. Reactivity of HIV-1 variants in diagnostic assays

The development of sensitive and specific tests to detect HIV-1 progressed rapidly after identification of this retrovirus as the causative agent of AIDS. However, it was not until continuous cell lines, permissive but resistant to cytopathic effects of HIV were discovered that manufacturers could produce enough viral antigen to make commercial kits (reviewed by Schochetman *et al*., 1989). In developing countries, ELISA's are the most commonly used tests for screening serum samples for the presence of HIV antibodies. Often, due to the cost of Western blot assays, a second ELISA assay based on a principle deviating from that of the first assay is used to confirm the screening results. In Cameroon, these tests are used mainly in reference laboratories. In smaller set-ups, rapid diagnostic assays which are less expensive, easier and faster to run are more commonly used. Such assays although easier to perform, have low sensitivity and specificity and therefore, need to be improved.

The high genetic variability observed among HIV strains affects antibody detection in serological assays. Although this effect is easily seen between the different groups, cases of non-detection of some group M viruses using reagents prepared with

viruses of this group have been reported (Huet *et al*., 1989; Montagnier *et al*., 1997; Baldrich-Rubio *et al*., 2001). These studies showed that atypical Western blots can be indicative of HIV-1 infection. The inclusion of group O peptides or viruses in different detection assays has recently increased both the specificity and sensitivity of these assays. Evaluation studies carried out shortly after the discovery of HIV-1 group O revealed the inability of ELISA used then to identify this group of viruses (Loussert-Ajaka *et al*., 1994; Gürtler *et al*., 1995). A similar situation is also currently being faced in monitoring the response to antiretroviral therapy in HIV-1 group O patients since no commercial tests for the quantification of this viral strain are available (Lerma *et al*., 2000). Sera from patients infected with HIV variants may be non-reactive or only weakly reactive in some commercial screening assays giving indeterminate WB patterns. The 99CMLB40 isolate in this study was in fact a complex AJU recombinant and might probably represent an antigenetically divergent strain. However, the fact that the envelope proteins were detected by Western blot argues against this. The absence of gag or very weak detection of pol proteins argues for an over all low level of antibody production by the infected patient. This patient was in fact at the terminal stage of AIDS and might have had at the time of sampling a complete break-down of her immune system accompanied by a selective loss of antibodies (Reimer *et al*., 1997).

### 4.2. Relatively fewer HIV-1-M subtypes circulate in Western Cameroon

Apart from sequencing which is the “gold standard” used for genetic subtyping, other methods like V3-loop peptide ELISA and the Heteroduplex Mobility Assay (HMA) have been widely used in HIV studies especially when dealing with a large sample size in regions where sequencing facilities are unavailable or limited (Delwart *et al*., 1993; Nkengasong *et al*., 1998). Discrepancies are however frequent between sequencing results and subtyping with V3-loop assays or HMA. One major limitation of HMA is its inability to distinguish recombinants from non-recombinants, especially when samples from areas where multiple genetic subtypes co-circulate are analysed (Nkengasong *et al*., 1998; Loussert-Ajaka *et al*., 1998; Heyndrickx *et al*., 2000). To produce reference reagents, it is therefore important to generate genomic sequences which could be phylogenetically analysed.

The presence of subtypes within the M group of HIV-1 was first suggested in 1992 on the basis of phylogenetic analysis of *env* gene sequences which revealed five clades approximately equidistant from each other (Myers *et al*., 1992). To date nine different subtypes are known designated A-D, F-H, J-K; and up to 11 different types of CRFs. The CRFs are recombinant forms which have evolved following recombination events involving two or more subtypes. The lack of subtypes E and I is indicative of the fact that some virus isolates had been previously assigned with these letters but later re-assigned as CRFs (Robertson *et al*., 2000). Subtypes differ in their geographical dissemination and have therefore been powerful molecular epidemiological markers for tracking the course of the global HIV pandemic (Peeters 2000).

It appears that the epicentre of the group M pandemic is in Central Africa, where the earliest known group M ancestral isolate was identified and where all currently identified group M subtypes are co-circulating with high levels of genetic diversity (Zhu *et al*., 1998, Nkengasong *et al*., 1994, Murphy *et al*., 1993; Vidal *et al*., 2000). In most countries of this region, HIV-1 genetic variability studies have been limited to major urban areas mainly because of easy accessibility to samples and laboratory facilities. In Cameroon for example, HIV genetic variability studies have so far been carried out in the central urban regions around Yaounde, the capital city and Douala where almost all HIV-1 groups and subtypes have been identified (Nkengasong *et al*., 1994; Takehisa *et al*., 1998; Tscherning-Casper *et al*., 2000a, Fonjungo *et al*., 2000). In this study, the 19 HIV-1 isolates characterised were obtained mainly from Western Cameroon. The study region included both, the North West and South West Provinces which lie between Central Cameroon and the neighbouring country Nigeria which shares vital socio-economic and linguistic ties with these provinces. While almost all known HIV subtypes have been found in the centre and large urban communities of Cameroon, Nigeria shows relatively fewer HIV-1 subtypes (Peeters *et al*., 2000).

In West and Central Africa subtype A viruses are the most prevalent with more than 50 % clustering with CRF02AG-like viruses (Heyndrickx *et al*., 2000, Montavon *et al*., 2000). These viruses cluster differently from subtype A viruses originating from East Africa (Carr *et al*., 1999). HIV prevalence studies from some West African countries indicate less variability in the circulating strains. Recently, Peeters *et al*. (2000) demonstrated in four main cities of Nigeria the dominance of HIV-1 group M viruses mainly A, G and CRF02.AG-like viruses. The panel of Western Cameroonian HIV-1

isolates were all group M with fewer HIV-1 subtypes when compared to previous studies in the central and northern regions of Cameroon (Takehisa *et al*., 1998; Nkengasong *et al*., 1994; Mboudjeka *et al*., 1999b). This should however be interpreted cautiously because of the relatively small number of study isolates and also since this is the first detailed molecular study of HIV isolates from this region of Cameroon.

Infections with more than one HIV subtype or group may lead to intersubtype or intergroup recombination (Takehisa *et al*., 1998; 1999). Intersubtype recombination occurs mostly in geographic areas where more than one subtype of HIV prevails. This has been shown to be relatively common in Central Africa (Heyndrickx *et al*., 2000, Vidal *et al*., 2000). Partial sequencing of the *env* region does not allow detection of these recombinant strains, while sequence analysis of regions from more than one of the structural genes (*gag*, *pol, env*) increases the probability of determining possible intersubtype recombinants (Heyndrickx *et al*., 2000; Cornelissen *et al*., 1996; Louwagie *et al*., 1993). Discontinuous portions from the 3 major structural genes of HIV were therefore analysed, allowing an estimation of possible non-recombinant or recombinant strains based on concordant or discordant phylogenies. The CRF02.AG-like strain was found to be the most prevalent subtype in this study accounting for roughly half of the isolates. Earlier studies on HIV-1 strains from Yaounde found a similar prevalence and CRF02.AG viruses have also been described as the most prevalent strain circulating in West and Central Africa (Heyndrickx *et al*., 2000, Montavon *et al*., 2000).

The two complete subtype J sequences currently available in the HIV data base have been obtained from the Democratic Republic of Congo, the former Zaire (Laukkanen *et al*., 1999) but recent studies have shown the spread of J sequences embedded in subtype A, G and CRF04.cpx sequences from Burkina Faso and other parts of West Africa and very recently also from Yaounde (Oelrichs *et al*., 1998; Tscherning–Casper *et al*., 2000a, Fonjungo *et al*., 2000). Unlike the recently described complex mosaic structure of an A/J/? strain from Botswana (Oelrichs *et al*., 1998) the *pol* gene of 99CMLB40 from this study is almost entirely of subtype J. Generally, the 99CMLB40 sequence showed a different pattern of clustering in the *gag-pol* region in relation to other J recombinants (AJ?, AGJ and CRF04.cpx) suggesting different ancestral recombination events. This isolate may therefore belong to a new lineage of CRF viruses circulating in Cameroon if at least one other epidemiologically

unlinked isolate with a similar recombinant structure is described after complete genome sequencing.

All subtype G viruses that have been completely sequenced so far contain portions of about 600 bp in the *vif* / *vpr* region which are either unclassified or subtype A (Gao *et al*., 1997; Carr *et al*., 1998) whereas the subtype G isolates obtained in this panel were complete G at least in the regions sequenced. Pure subtype G sequences are required to define the parental forms of the dominant CRF01.AG circulating in West and Central Africa (Carr *et al*., 1998). Subtype F viruses were recently reclassified into subtype K and sub-subtypes F1 and F2 (Robertson *et al*., 2000). These viruses which have been predominantly reported in Cameroon with prevalence rates ranging from between 7-17% are also present in other parts of Africa, South America, and Europe (Takehisa *et al*., 1998; Nkengasong *et al*., 1994; Peeters *et al*., 2000)

Conspicuously absent in this panel of isolates were HIV-1-M subtypes D, H, CRF02.AE and HIV-1-O. These viral forms have been previously described with a high degree of frequency from Cameroon (Nkengasong *et al*., 1994; Mboudjeka *et al*., 1999b; Takehisa *et al*., 1998). Considering the fact that the most divergent forms of HIV-1, SIV and also other human retroviruses like the human T-cell leukaemia virus (HTLV) were first, or are commonly described from central Cameroon (Simon *et al*., 1998; Mahieux *et al*., 1997, Takehisa *et al*., 2001; Van Dooren *et al*., 2001), it is tempting to speculate that the centre of HIV diversity might be located towards the central region of Cameroon. This might imply that, as one moves from central Cameroon towards Nigeria in the West of Africa, the number of circulating viral subtypes and therefore viral diversity reduces. One might speculate however, that within years to come, a more even distribution of viruses may occur.

### 4.3. Drug resistance signal sequences among drug naive individuals

As mentioned earlier, most HIV-1 infections worldwide have been caused by group M viruses, and consequently most information on the course of infection have been gathered on this group. Particular bias regarding studies on drug susceptibility and resistance has been on subtype B viruses which is rare in Africa, but is the prevalent subtype in countries with access to antiretrovirals. The protease and reverse transcriptase which are the main antiviral targets have been widely studied. Currently few sequence data are available for the protease and reverse transcriptase genes of

non-subtype B isolates, but some HIV-1 subtype G, F and group O viruses and also HIV-2 have been shown to be less susceptible to respective inhibitors (Descamps *et al*., 1997; 1998; Apetrei *et al*., 1998). The isolates described in this study were obtained from drug-naive HIV infected individuals. None of the isolates showed amino acid compositions in the respective genes known to directly cause drug resistance. The protease M36I exchange has been associated with inhibitor resistance in the presence of additional signal sequences (Condra *et al*., 1995). This M36I polymorphism which contributes to nelfinavir and ritonavir resistance appears to occur frequently among non-B isolates. Cornelissen *et al*. (1997) found a high prevalence of the M36I substitution (71 %) in a panel of subtype A-D isolates and also that the protease gene was more variable than the 5' half of RT; suggesting that the protease enzyme might be more flexible in tolerating substitutions in critical positions. Recent *in vitro* susceptibility tests with subtype C viruses from Zimbabwe did not show any decrease in drug sensitivity despite the presence of accessory polymorhpisms in the protease gene (Shafer *et al*., 1999), indicating that accessory polymorhisms are not always associated with drug resistance/susceptibility.

The low prevalence of primary drug resistance signal sequences in these isolates is consistent with the absence of widespread use of antiretroviral medication in Cameroon. It is not known whether drug resistance polymorhisms characterised in subtype B HIV-1 have the same phenotypic effects in other HIV-1 subtypes. At a time when antiretrovirals are being introduced in developing countries and viral strains from the developing countries are being increasingly reported in Europe and America such studies are needed to better understand the biological implications of such sequences.

### 4.4. HIV-1 biological phenotype correlates with co-receptor usage

HIV-1 isolates display different tropism for various cell types. After the discovery of the co-receptors for both HIV and SIV, it became evident that the co-receptors expressed on the surface of cells play an important role in HIV entry. The ability of the HIV envelope protein to interact with a specific co-receptor determines the capability of a virus to infect these cells. Several studies have shown a broadening of co-receptor usage profile of HIV-1 isolates associated with progression to AIDS and about 50 % of viruses derived from AIDS patients showed usage of multiple co-

receptors (Hoffman and Doms, 1998). The usage of more than one co-receptor for entry could be due also to the presence of a heterogeneous virus population in the primary isolate each using a different co-receptor. The only possible means of excluding this is by biologically cloning or by generating molecular clones of the envelope region from an isolate and testing for co-receptor usage. Extensive biological characterisation of HIV-1 isolates have been performed mainly on subtype B isolates or a limited number of HIV-1 strains of other subtypes which have been passaged in T-cell lines. This study is the second to determine co-receptor usage of HIV-1 strains from Cameroon, the first study concentrated on isolates from pregnant women in Yaounde (Tscherning-Casper *et al*., 2000b). Studies on the probable association between HIV-1 subtypes and co-receptor usage carried out recently indicate that although co-receptor usage is independent of genotype, there might be exceptions (Tscherning *et al*., 1998). One of these studies reported the exclusive usage of CCR5 by subtype C viruses and the rare occurrence of dual tropic subtype D viruses. However only the ability of subtype C viruses to use CCR5 and not CXCR4 has been confirmed in other recent studies (Bjorndal *et al*., 1999; Cecilia *et al*., 2000), although the reason for this preferential tropism is not known. The main co-receptors used by different HIV-1 isolates for entry are CCR5 or/and CXCR4 (Bjorndal *et al*., 1997). Additional members of the chemokine receptor family and several other seven transmembrane domain receptors also serve as co-receptors for HIV and SIV in vitro, although their importance *in vivo* remains unclear (Hoffman and Doms, 1998). In a recent co-receptor study, Tscherning-Casper *et al*. (2000b) described the usage of the orphan co-receptor Bonzo (commonly used by SIV and HIV-2, Enders *et al*., 1996) by four HIV-1 isolates from Cameroonian pregnant women and one from a new-born baby. These authors suggested that Bonzo may be used frequently by *env* subtype A and CRF02.AG viruses. However, none of the 14 isolates from this panel, which were either subtype A or of the CFR02.AG lineage in *env*, was able to use Bonzo. Therefore unlike subtype C viruses which preferentially use CCR5 independent of the patient's disease status, Bonzo usage does not appear to correlate with HIV-1 genotype (Tscherning *et al*., 1998). In fact, co-receptor usage by all of the isolates in this Cameroonian panel was dependent on biological phenotype and not genotype, thus supporting previous studies (Bjorndal *et al*., 1997; Tscherning *et al*., 1998, Zhang *et al*., 1996).

### 4.5. The V3 loop as a determinant of HIV-1 tropism and co-receptor usage

In addition to playing a role in determining cellular tropism, the V3 loop of gp120 determines co-receptor usage for viral entry (Cocchi *et al*., 1996, Trkola *et al*., 1998). When the V3 region of SI infectious molecular clones are replaced with sequences from NSI isolates, this results in a switch in co-receptor usage from CXCR4 to CCR5 (Cocchi *et al.*, 1996). Also, successive passages of HIV-1 resulting in specific changes in the V3 loop structure have been associated with changes in viral phenotype (De Jong *et al*., 1992; Fouchier *et al*., 1992). The V3 sequences of NSI isolates representing the various genotypes were more conserved compared to those of the SI isolates. The variability observed within the SI isolates perhaps reflects viral adaptation to host immune responses since SI isolates appear at a later stage of disease. Several studies have identified basic amino acid residues at two to four positions within the V3 loop that alter tropism and phenotype (Bhattacharya *et al*., 1996; De Jong *et al*., 1992; Fouchier *et al*., 1992). Others have however argued that the overall charge and not particular amino acids on either side of the GPG tip of the V3 loop is responsible for the tropism and phenotype (Chesebro *et al*., 1992, 1996; Shioda *et al*., 1992). Most of these studies have been carried out with laboratory adapted strains of HIV-1 which have been shown to differ significantly from their progenitor patient isolates. For example they show altered sensitivity to neutralisation and infect a variety of T-cell lines (Kabat *et al*., 1994). Using this Cameroonian panel of HIV-1 isolates it is clear that there are exceptions to both hypotheses of overall charge and location of particular amino acid residues in the V3 loop as determinants of viral tropism.

### 4.6. Recombinant structure of A/G and A/J viruses from Cameroon

Initial reports on HIV-1 recombination indicated that at least 10 % of HIV-1 sequences in the database were possibly intersubtype recombinants (Robertson *et al*., 1995). Recent studies suggest that this number might be even higher (Heyndrickx *et al*., 2000; Montavon *et al*., 2000). Full-genome sequencing as well as new reliable methods for analysing these sequences such as similarity plots and bootscanning methods have greatly facilitated the process of identifying recombinant viruses. The value of full-genome analysis has been repeatedly demonstrated. For example viral

strains causing the HIV-1 epidemic in Thailand, previously classified as “subtype E” were later found to be recombinant viruses between a supposedly extinct “subtype E” and subtype A. They are now termed CRF01.AE (Robertson *et al*., 2000). Also one of the earliest identified HIV-1 strains, Z321 from the democratic Republic of Congo used for the production of candidate HIV-1 vaccines was first thought to be subtype A based on envelope sequence. Later after full-genome analysis it was found to be an A/G recombinant (Choi *et al*., 1997).

By using the full-genome sequencing approach the entire genome of BD6.15 was screened and the recombination cross over points identified. BD6.15 is a CRF02.AG strain since it clusters with other CRF02.AG phylogenetically and represents the most dominant strain of HIV-1 circulating in Cameroon (Heyndrickx et al., 2000, Montavon *et al*., 2000).

Studies have shown that some viruses belonging to the same or different CRFs may share some recombination crossover sites. This is the case with CRF01.AE which all show the same mosaic pattern indicating that they were probably derived from a common ancestor (Gao *et al*., 1996; Carr *et al*., 1996). CRF02.AG viruses on the other hand seem to be more heterogeneous in the distribution of their crossover points. The most dominant form however is the IbNg form which is believed to have been the most successful progeny of all ancestral CRF02.AG recombinants (Carr *et al*., 2001; Montavon *et al*., 2000). The IbNg virus obtained from a 23 year old asymptomatic HIV-1 individual from Ibadan, Nigeria, was the first A/G recombinant to be described (Olaleye *et al*., 1996; Howard and Rasheed, 1996). The CRF02.AG clone, BD6.15 shares the same pattern of break points with other CRF02.AG especially the IbNg strain. Carr *et al*. (2001) also recently reported the presence of A/G recombinants and some novel recombinants of HIV from the eastern and central regions of Cameroon.

The A/J crossover point of the 99CMLB40 isolate was located at the *gag-pol* boundary similar to that of CRF02.AG viruses. Other studies which have also reported subtype J sequences from Cameroon did not include detailed genome analysis (Tscherning-Casper *et al*., 2000a; Funjungo *et al*., 2000), therefore it is difficult to conclude if this recombinant structure of 99CMLB40 is frequent in Cameroon. At first sight it may seem that there could be a possibility of recombination “hotspots”. These are genomic regions where recombination occurs at high frequency. They include secondary structures or regions where there is a certain

level of sequence identity favouring recombination. Although a few studies have described similar crossover points between different strains (Baldrich-Rubio *et al*., 2001), an increasing number of fully sequenced viruses does not support this hypothesis. Even studies using two different molecular clones of HIV-1 subtype B to determine possible recombination hotspots concluded that recombination seems to occur at high rates but with equal frequency along the entire HIV genome (Jetzt *et al*., 2000). It is however interesting to note that CRF02.AG recombinants described so far usually tend to have a "preference" of specific subtypes for specific genes (Carr *et al*., 2001). Whether this portrays an ancestral relationship between these viruses or a selective advantage of having certain genes from certain subtypes, remains to be elucidated. The CRF01.AE and CRF02.AG, the two most wide spread forms of any CRFs described to date are widely believed to be likely a result of a founder effect, that is a single infection having spread from one source. Recently two "second generation" recombinants composed respectively of a CRF02.AG / subtype C and CRF02.AG / subtype G were described (Janssens *et al*., 2000), indicating that CRFs may still recombine with other subtypes to generate even more complex mosaic viruses. Monitoring the frequency and complexity of HIV-1 recombinants and also the clinical status of infected individuals are important goals towards better understanding the AIDS epidemic.

### 4.7. Group O viruses are the most genetically diversified form of HIV-1

Group O viruses were first described in 1990 and fully characterised four years later (De Leys *et al*., 1990; Gürtler *et al*. 1994; Vanden Haesevelde *et al*., 1994). The earliest case of disease caused by these viruses was reported from sequence analysis of Norwegian patients believed to have been infected in the early 1960's (Jonassen *et al*., 1997). The epicentre of group O infection is in Central Africa. However, viruses of this group have also been reported in some other parts of Africa, in Europe and the United States (Peeters *et al*., 1997; Hampl *et al*., 1995; Rayfield *et al*., 1996). It became clear that group O viruses were distinct from other HIV's when sera from patients presenting with clinical symptoms of AIDS completely escaped detection or were only weakly reactive to HIV-1/2 antibody assays used at that time (Loussert-Ajaka *et al*., 1994). The recent modification of HIV-1 commercial ELISA kits

by the inclusion of group O peptides has greatly improved their sensitivity to group O viruses.

Despite their similarity in genomic arrangement, the genetic distances between group M subtypes and group O viruses are quite significant. Nucleotide sequence diversity between these two groups in *gag*, *pol* and *env* ranges from 24 to 32%, 33 to 37% and 39 to 49% respectively (Descamps, *et al*; 1995; Loussert-Ajaka *et al*., 1995). To date no difference has been noticed in the pathogenic course of individuals infected with either HIV-1 group M or the outlier viruses. These observations are preliminary since only two follow-up cases of HIV-1-O infections have been documented (Nkengasong *et al*., 1997).

Most studies on group O viruses have been limited to molecular analysis of the structural genes (*gag*, C2V3 region of *env*) and accessory genes (Loussert-Ajaka *et al*., 1995; Brennan et *al*., 1997; Hackett *et al*, 1997; Bibollet-Ruche *et al*., 1998). Sequences from these regions do not show any distinct clustering pattern as has been found among group M subtypes implying that partial analysis of selected regions of the genome are not informative enough to reveal detail phylogenetic relationships. In fact two studies have attempted to classify group O viruses based on the partial genome sequences of *env* and *gag* (Mas *et al*., 1999, Janssens *et al*., 1999). These partial analyses coupled with the fact that group O primary isolates are not readily available are reasons for the limited information about this group of viruses. Until very recently, only two full genome sequences of group O viruses were available. The generation and complete sequencing of more HIV-1-O molecular clones may facilitate the phylogenetic classification of this group of viruses.

Phylogenetic analysis of partial *gag* and *env* group O sequences from the HIV database, show that most of the group O viruses isolated to date are closer to ANT70 than to MVP5180 (Quinones-Mateu *et al*., 1998; Kane *et al*., 2001). This was also observed with the CMH2.3 clone when its full genome sequence and complete sequences of *gag, pol, env* and *nef* were separately analysed. Therefore this clone is a true representative of the most dominant form of circulating HIV-1-O strains.

To date, few studies have attempted to unveil the possible differences that might exist between HIV-1 group O and M at the level of the primary isolate. A study by Braaten *et al.* (1996) showed that the HIV-1-O isolate MVP 5180 incorporated the cellular protein cyclophilin A like members of the group M but infectivity was not impaired by cylosporin treatment. However, recent studies with a panel of HIV-1

group O isolates indicate that all do incorporate cyclophilin A, however a majority but not all are sensitive to cyclosporin treatment (Wiegers and Kräusslich, in press). Studies of co-receptor usage by primary HIV-O isolates reveal that most isolates obtained at late stages of AIDS still use CCR5 for entry (Dittmar *et al*., 1999). Serum from HIV-1-O patients usually lack cross neutralisation antibodies to HIV-1-M and also, isolates of HIV-1-O show complete resistance to TIBO nucleoside analogues and to nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI) widely used in reducing mother to child transmission of HIV (Descamps *et al*., 1995)

The epidemiological importance of group O viruses was shown more recently when two independent studies identified a recombinant O/M virus from two Cameroonian patients (Takahisa *et al*., 1999; Peeters *et al*., 1999). Both O/M recombinant strains replicated well *in vivo* and *in vitro* and one of them even became the predominant variant within the patient. This finding indicates that although group O viruses are still limited mostly to West and Central Africa, they could as well spread out to other regions of the world in the form of recombinants as is currently the case with group M viruses.

The PCR strategy employed to amplify the entire proviral genome aiming at producing infectious DNA was necessary due to the redundancy of the LTRs. The method was first established to amplify the entire genome of a foamy virus isolate with a proviral genome size of more than 13.000 base pairs (Herchenröder *et al*., 1995). To circumvent the LTR-redundancy problem most studies to date have concentrated on the generation of virtually full-length molecular clones representing different subtypes of HIV-1. Long PCR fragments stretching from the U5 region of the 5’ LTR to the R region of the 3’ LTR, or from the primer binding site to the 3’LTR were first cloned into a vector and then the 5’LTR was sub-cloned subsequently (Salminen *et al*., 1995b; 2000; Dittmar *et al*., 1997b). The results described here once more prove that by introducing silent mutations in PCR primers that hybridize to HIV sequences outside of *cis*-acting elements and subsequent “chessboard” cloning, full-length infectious molecular clones can in fact be generated.

Also since a complete genome might encode several defective genes, amplification of the entire genome in two halves and recombining different clones increases the chance of obtaining a non-defective full-length infectious clone.

### 4.8. Infectious molecular clones as reference reagents

In order to study the biological, immunogenic and pathogenic properties of different HIV subtypes, well-characterised virological reference reagents, in particular full-length infectious clones are a necessary prerequisite. To date optimized molecular reagents for viruses other than subtype B are quite rare or in some cases non-existent. Of the few full-length infectious molecular clones that exist, only three of them represent HIV-1 subtypes C, CRF01.AE and CRF02.AG which play dominant roles in the pandemic (Salminen *et al*., 2000; Ndung'u *et al*., 2001; Takahoko *et al*., 2001). In this study two new full-length infectious molecular clones representing HIV-1 group O and the CRF02.AG were generated and characterised. The clone CMH2.3 represents the first HIV-1 group O infectious molecular clone while the clone BD6.15 is the second CRF02.AG infectious molecular clone but the first CXCR4 representative to be produced.

The difference observed in replication between the HIV-1-O primary isolate and the trasfection derived provirus in PBMCs could be due to two reasons. One possible explanation is that this clone which was used for tranfection might represent just a minor variant in the swarm of viruses found in the primary isolate which do not replicate well in PBMCs, compared to other dominant variants in the primary isolate. The second possibility is the defective *vpr* in this clone that is caused by an in-frame stop codon observed after the entire CMH2.3 clone was sequenced (section 3.2.2). The *vpr* of HIV has been reported as one of the proteins that targets the pre-integration complex to the nucleus, thereby enabling infection and efficient replication in non-dividing cells such as terminally differentiated macrophages (Balliet *et al*., 1994). It has also been reported that HIV-1 mutants lacking *vpr* replicate less effectively in non-dividing macrophages than wild type HIV-1 mutants (Vodicka *et al*., 1998), however this data has not been supported by others (Reil *et al*., 1998; Bouyac-Bertoia *et al*., 2001). The fact that virus derived from this clone uses CCR5 as co-receptor and that CCR5 viruses preferentially infect macrophages might argue for a possible role by *vpr* in this reduced replication in PBMCs. The identification of another variant without a stop codon in the primary isolate (section 3.2.2) may therefore explain its better replication capacity in PBMCs. However, since replication kinetic experiments were not done on macrophages with the CMH2.3 clone, it is

premature to conclude that the poor replication of this clone in PBMCs was linked to this stop codon.

Both viruses from which the infectious molecular clones were derived originated from Cameroon where one of the highest rates of HIV genetic variability has been reported. The effort must be valued under the context that infectious molecular HIV clones are difficult to generate due to the large number defective genomes present in HIV infected individuals. These clones will enhance efforts aimed at understanding the biological consequences of HIV genetic diversity and its impact on cellular and immune response in its host. They could also be important in identifying cross-clade specific CTL epitopes in preparing protein-based subunit vaccines as well as cocktails of genetically diverse immunogens. The full-length sequences will be useful in phylogenetic analysis of HIV subtypes originating from West Central Africa.

In particular, CMH2.3 representing the slow but continuous spreading HIV-1-O will serve as a useful tool to elucidate some difference between this group and HIV-1 group M viruses such as co-receptor usage, incorporation and dependence on the cellular factor cyclophilin A, and potential resistance to some anti-retroviral drugs which are gradually being made available in Africa, the centre of the HIV pandemic.

Immunogens to be included in any future vaccine should be antigenically close to the most prevalent strain(s) circulating in a geographical region. Based on this study and those of others, there is certainly the need to include at least CRF02.AG based immunogens into any experimental vaccine to be used in Cameroon.

### 4.9. Implication of recombination and subtypes in the HIV global epidemic

Replication efficiency and modifications in tropism are some of the added advantage recombinant viruses might have over their parental strains. It has been suggested that the dominant CRFs in the HIV pandemic might have an added advantage of fitness over their parental isolates, therefore explaining their high prevalence. The dominant CRFs described so far include, CRF01.AE in Thailand, CRF02.AG in West and Central Africa, CRF03.AB in Kaliningrad and the B/C recombinants in China (Heyndrickx *et al*., 2000, Motavon *et al*., 2000; Su *et al*., 2000; Liitsola *et al*., 1998). However, this point has not been proven beyond epidemiological data. *In vitro* experiments with feline and murine retroviruses have shown that mixed infections can generate recombinant viruses with altered tissue tropism, pathogenicity and

antigenic properties (Tumas et al., 1993; Golovkina et al., 1994). Also, under selective pressure of antiviral drugs, recombination of variants with different drug sensitivity have led to the emergence of variants with dual drug resistance (Moutouh et al., 1996). On the other hand, the global distribution of HIV-1 subtypes from which these recombinants are generated is uneven and diversity may even reach 24% in Env. Such high diversity suggests the possibility of functional or biological differences between subtypes. In fact some epidemiological studies have suggested that HIV-1 subtypes may differ in selected properties. Examples include: the predominant usage of the chemokine receptor CCR5 by HIV-1 subtype C (Bjorndal *et al*., 1999), a more rapid progression to AIDS by women infected with non-subtype A than those with subtype A viruses in Senegal, West Africa (Kanki *et al*., 1999), and association of HIV-1 genotypes with differential possibilities of perinatal transmission in Tanzania, East Africa (Renjifo *et al*., 2001). All these point to the fact that specific HIV-1 recombinants and subtypes may have different effects in the spread and control of HIV.

### 4.10. Future perspectives

The evolution of HIV is a dynamic process and requires regular monitoring for emerging variants. Such “new” viruses might have an impact on virus detection, pathogenesis, drug resistance and vaccine development. Epidemiological studies suggest subtype specific differences of HIV strains. These can only be confirmed either through *in vitro* or *in vivo* follow-up studies in animal models using infectious molecular clones representing different subtypes.

One of the *in vitro* studies suggested is the determination of viral factors such as amino acid residues important for the preferential usage of CCR5 and not CXCR4 by HIV-1-O isolates. Studies of this sort will require generating recombinant *env* molecular clones involving HIV-1-O isolates and the full-length clone described in this study.

Also planned are studies to determine the specific amino acid mutations in protease and RT which may influence drug resistance by HIV strains circulating in Cameroon. To carry out such studies, it is important to understand the distribution of circulating viral strains in a population. However, since an HIV subtype distribution map of Cameroon already exists, it will now be necessary to generate recombinant HIV-1 *pol*

clones which will be used to monitor possible drug resistance strains in phenotypic assays. Studies of this sort are of importance at this point in time when the administration of anti-viral medication is being increased in Cameroon and the neighbouring countries.

## 5. SUMMARY

Human immunodeficiency virus (HIV) displays a high degree of genetic variability, which is mainly attributed to its high replication rate and the error prone nature of the viral reverse transcriptase. This genetic variability necessitated its classification into two types (HIV-1 and –2), each subdivided into several groups and subtypes. Even within a single infected host, many genetically different viruses exist and this swarm of sequences has been termed viral quasispecies. Furthermore, the genomes of viruses of different subtypes and groups may recombine to yield circulating recombinant forms (CRF), some of which show a high prevalence in certain areas of the world. Quite clearly, the current AIDS pandemic is caused by a large number of genetically different viruses, which are originally derived from few cross-species transmissions, but have evolved and are continuing to evolve at a rapid rate. Currently, there is little known about potential functional differences between the various subtypes and recombinant forms. Most studies have been performed with laboratory strains of the virus and few reagents directly derived from the primary patient-derived virus are currently available. To study specific differences between various strains and recombinant forms, one needs to obtain primary isolates of the virus reflecting the viral quasispecies within a given person. Detailed molecular analysis of the functional properties and biological phenotypes of specific viruses further requires the availability of full length molecular clones of the respective viral genomes. Transfection of such proviral clones leads to production of infectious virus particles, whose properties should be identical to the primary isolate the clone was derived from. It was the aim of this study to obtain a panel of primary HIV isolates from Cameroon and to generate infectious molecular clones derived from relevant viral variants.

Blood samples were collected from HIV-1 infected individuals mostly from the Western Provinces of Cameroon. A total of 19 HIV-1 isolates were derived from these samples by co-cultivation of patient-derived blood cells with either peripheral blood mononuclear cells from HIV negative donors or with PM-1 cells. PM-1 is a permanent T-cell line, which expresses the two main coreceptors of HIV-1 (CXCR4 and CCR5), and therefore allows propagation of most viral isolates, irrespective of their coreceptor usage. Genetic subtyping of the virus isolates was performed by PCR amplification and direct sequencing of discontinuous portions of the three major

structural genes *gag*, *pol* and *env*. Sequences were phylogenetically analysed and classified into the following HIV-1 main (M) group subtypes: $A^{gag}/A^{pol}/A^{env}$ (n=4); $G^{gag}/G^{pol}/G^{env}$ (n=3), $F2^{gag}/F2^{pol}/F2^{env}$ (n=1), $A^{gag}/AG^{pol}/AG^{env}$ (n=2), $AG^{gag}/A^{pol}/AG^{env}$ (n=1), $AG^{gag}/U^{pol}/AG^{env}$ (n=1), $AG^{gag}/AG^{pol}/AG^{env}$ (n=6) and a novel $A^{gag}/J^{pro/rt}/A^{int}/U^{env}$ complex recombinant (n=1). Thus, only few viral isolates displayed a pure subtype and there was a high prevalence of recombinant forms, mostly corresponding to the CRF02.AG type. Two recombinant strains were selected for further analysis: An AJU recombinant because it is the first isolate of this kind to be reported from Cameroon and a CRF02.AG isolate because this is the most prevalent HIV-1 strain currently circulating in West and Central Africa. While the recombinant structure of the AJU virus was different from other subtype J recombinants, the CRF02.AG isolate was very similar to other viruses of this lineage.

The biological phenotype of most isolates correlated with the clinical status of the patient. Five of six isolates from patients with advanced disease induced syncytia (SI) on MT-2 cells while 13 isolates, 12 of which were from patients at earlier stages of the disease, were of the non-syncytium inducing phenotype (NSI). All SI isolates used CXCR4 as their co-receptor, while all NSI isolates used CCR5. Sequencing of the V3 region within the envelope gene revealed additional feature which had been attributed to the respective biological phenotype in previous studies. To determine whether drug resistance mutations were present in these isolates, which had been obtained from drug-naive individuals, the protease and part of the reverse transcriptase coding region was screened for known resistance polymorhisms. However, only variations considered to be compensatory polymorhisms were observed and no primary resistance signal sequence was detected.

In order to obtain reference tools for HIV research, the complete genomes of an HIV-1 group O strain and of a CRF02.AG isolate were amplified by long range PCR and full-length molecular clones were generated. Production of infectious virus was detected after transfection and infectious molecular clones were completely sequenced. Transfection-derived viruses exhibited comparable biological properties and infectious titers as their respective parental isolates. The HIV-1 group O proviral plasmid represents the first infectious molecular clone of this group of HIV-1, and the CRF02.AG clone is the first CXCR4-using infectious clone from this highly prevalent recombinant variant. These reagents will be very important for further studies aimed

at elucidating the biological and functional differences that might exist between HIV subtypes. In addition, they may serve as valuable tools in HIV vaccine studies.

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# 7. Appendix

## 7.1 Primers

| Primer | Sequence (5'-3') | Orientation | Location in HXB2 |
|---|---|---|---|
| **HIV-1-M** | | | |
| Gag1[a] | tatgggtgcgagagcgtcggtattaag | sense | 789-815 |
| gag2[a] | tttggtccttgtcttatgtccagaatgc | antisense | 1631-1658 |
| gag3[a] | tgggaaaaattcggttaaggcc | sense | 836-857 |
| gag4[a] | tactattttatttaatcccaggattat | antisense | 1585-1612 |
| pol1[a] | tcaagggaaggccagggaattt | sense | 2111-2132 |
| pol3[a] | taatattgctggtgatcctttcc | antisense | 3007-3029 |
| pol2[a] | tcctttaacttccctcaaatcact | sense | 2241-2264 |
| pol4[a] | tacattgtactgatatctaatccctggtgt | antisense | 2964-2993 |
| Env1[a] | tagcacagtacaatgtacacatggaat | sense | 6950-6976 |
| Env2[a] | gtgcttcctgctcccaagaaccca | antisense | 7784-7807 |
| Env3[a] | tgttaaatggcagtctagcagaa | sense | 7003-7025 |
| Env4[a] | ttatataattcacttctccaatt | antisense | 7656-7678 |
| Mlubase1[b] | gggcccacgcgtgatgggttaatttactccaagaaaagacaaga | sense | 4-40 |
| Revsal2[b] | gagttctctgaagtcgactaattttctcca | antisense | 2760-2789 |
| Forsal3[b] | tggagaaaattagtcgacttcagagaactcaataaaag | sense | 2760-2797 |
| Notlast[b] | agtcgcggccgcggtctgagggatctctagttaccagagtc | antisense | 9662-9702 |
| B4PBS- | ctgtgtgcacctcagcaagccgag | antisense | 695-719 |
| mtd1 | aggggccaagtcggcctctctagcagtggcgcccgaacaggg | sense | 625-651 |
| gag1revM | gtgcgaatctttccaactccctgct | antisense | 901-925 |
| MidgagM | agccaaaattaccctatagtgc | sense | 1174-1195 |
| gag5forM | ttaccctatagtgcaaaatgcacaag | sense | 1180-1204 |
| ca 3a-kurz | aggaactactagtacccttcagga | sense | 1500-1523 |
| gag6forM | tgagtcaagtacaacaggcaggag | sense | 1124-1147 |
| pol5forM | gttgactcagattggttgcactct | sense | 2519-2549 |
| 3'ProteaseM | gacgaaatatgttgactcagattgg | sense | 2509-2533 |
| pol6forM | gttaagagaacatctactgagatg | sense | 3161-3184 |
| SE3446 | gagattctaaaagaaccggtacatggag | sense | 3471-3498 |
| seqrtrev | ggctcttgataaatttgatatgtccattg | antisense | 3555-3583 |
| pol7forM | ctagtaaaattatggtaccagtta | sense | 3813-3836 |
| SE4170 | ctacctggcatgggtaccagcacacaaag | sense | 4142-4147 |
| SE4142 | ctttgtgtgctggtacccatgccaggtag | antisense | 4142-4147 |
| pol8forM | gctatatagaagcagaagttatcc | sense | 4426-4449 |
| P1[c] | ccctacaatccccaaagtcaagg | sense | 4653-4675 |
| P2[c] | tactgccccttcacctttcca | antisense | 5253-5277 |
| midvifM | tctgcaaacaggagaaagagactgg | sense | 5253-5257 |
| 5'VPR | agctgcaggtaccatggaacaagccccag | sense | 5546-5574 |
| 3'VIF | agctagtgtccattcattg | antisense | 5603-5621 |
| ED3 | ttaggcatctcctatggcaggaagaagcgg | sense | 5957-5986 |
| vpu1 | tattggggtctgtgggtacacagg | antisense | 6442-6465 |

| | | | |
|---|---|---|---|
| ENVC | ttgtgggtcaccgtctattatgggg | sense | 6324-6348 |
| MidEnvM | tctttaataaatcctcaggagg | sense | 7303-7324 |
| EnvC2-M | ctgcactttcaatacgaccacag | sense | 6692-6714 |
| 7029+ | tggcagtctagcagaagaag | sense | 7010-7029 |
| Env7forM | ctaaatcctcaggaggggatgtag | sense | 7298-7322 |
| env5 | tcagacctggaggaggagatatga | sense | 7627-7650 |
| env6 | tgaagaatcgcagaagcagcaaga | sense | 8162-8185 |
| gp41F | taaatagagttaggcagggata | sense | 8338-8359 |
| neffor | atgggtggcaagtggtcaaaaa | sense | 8797-8818 |
| 3'LTRfor | aggtacctttaagaccaatgac | sense | 9014-9035 |
| nef-7 | agcagttcttgaagtactccg | antisense | 9395-9415 |
| | | | |
| **HIV-1-O** | | | **Location in Ant 70** |
| base1ant70[b] | ttctcgagtcgactggaagggttaatttactccc | sense | 1-22 |
| MLUrev2[b] | ttgtcccaacgcgtgtagccaaggcctaggg | antisense | 5714-7544 |
| MluIfor[b] | tggctacacgcgttgggacaatac | sense | 5724-5747 |
| last-ant70[b] | ttccgcggccgctgctagagattttctgcttcagtc | antisense | 3‘end 3‘LTR |
| M13rev[c] | caggaaacagctatgaccatg | sense | vector |
| M13for[c] | gtaaaacgacggccagt | antisense | vector |
| 5'U3F-O | tgtgggactttccaacagagactg | sense | 365-388 |
| 5'LTRfor | aggtacctttgagaccaatgac | sense | 9099-9120 |
| Mat for O | gctacagcagttagagccagctctcaagac | sense | 1021-1050 |
| Caprev-O | ctatccattttctatagatgtctcctactg | antisense | 1608-1638 |
| capfor-O | gaagcagtagagtgggatagaact | sense | 1457-1480 |
| cap2for-O | ttcatgcaaagagggcaaaatcca | antisense | 1982-2005 |
| 3'Protease-O | gaacagtattggtgggacctactc | sense | 2525-2548 |
| ProteaseO/M[c] | aaaacctcctatacccctatcatttttgg | antisense | 2437-2466 |
| salfor-O | gagaaaattggtcgactttagggaattaaat | sense | 2817-2847 |
| salrev-O | ttaattccctaaagtcgaccaattttctcca | antisense | 2815-2845 |
| 5'RT-O | ttcctagtgtgaataatgaga | sense | 2999-3019 |
| MidRT-O-3 | gagaaaagctaaaggagccagtgc | sense | 3524-3547 |
| RT-O-4 | gtgaacctattatgggggcagaaa | sense | 3896-3919 |
| IntF-O | ctttatgggcaggaacccatgtaa | sense | 4202-4225 |
| IntrevO/M[c] | tgatatttttcatgatcttcttg | antisense | 4309-4331 |
| P-O-3 | ctgcctacttcctgttaaaattag | sense | 4574-4597 |
| vifend | catagtcaggtagggacacta | sense | 5509-5529 |
| vif1 | tcgggtttattacagggacagcagag | sense | 4952-4977 |
| 3'VIF | agctagtgtccattcattg | antisense | 5657-5675 |
| 5'rev-O | atctcctatggcaggaagaagc | sense | 6019-6040 |
| Env-O-5' | ctatatgcaacagtctatgctgggg | sense | 6371-6395 |
| rev-tatO/M[c] | tacttactgctttggtacagga | antisense | 6081-6102 |
| Env1-O | ccaattccaatacactattgtgctc | sense | 6893-6917 |
| Env1-Orev | gttggcctaatgccatgtgtacaag | antisense | 6996-7020 |
| EcoRIrev-O | gaatgttaagaattcctcttcctatccttt | antisense | 8792-8821 |
| EcoRIfor-O | tcatagtgaccctgaattctactgtaaacat | sense | 7110-7140 |
| Env2-O | caaacagctgaaaggtattt | sense | 7289-7308 |
| Env2-Orev | cctactgctctttttctctatgag | antisense | 7794-7818 |

| | | | |
|---|---|---|---|
| Env3-OHD | ttgggaaaacctaacatggcagca | sense | 8136-8159 |
| gp41R-O | gagaggggttgatatccctgcctaa | antisense | 8393-8417 |
| pptrev-O | cttttcttttaaaaagaagctgag | antisense | 9140-9163 |
| 3'LTR-O | taagacctcaagtgcctctaaggc | sense | 9090-9113 |
| 5'U3Fnew | gaagatggctgccgctgtggg | sense | 381-404 |
| Nefstart-O | atggaaaatgcattgaga | sense | 8864-8881 |

**Table 6.1:** Primers used for PCR and sequencing reactions.

[a] = short fragment PCR primers; [b] = long range PCR primers; [c] = primers used to sequence both HIV-1-goups M and O.

Nucleotide bases: a = adenine, t = thymine, g = guanine, c = cytosine

### 7.2 PCR and sequencing programs:

Short fragment PCR: 94°C for 2 min, 94°C for 15 sec, 53°C for 45 sec (x40), 72°C for 5 min.

Long PCR: 94°C for 2 min (1x), 94°C for 15 sec, 56°C for 30 sec, 68°C for 6 min (30x) and 72°C for 30 min.

Cycle sequencing: 96°C for 40 sec, 50°C for 40 sec, 60°C for 4 min (30X).

### 7.3 Abbreviations

| | |
|---|---|
| A | Adenine |
| AIDS | Acquired immune deficiency syndrome |
| bp | base pairs |
| °C | degree Celcius |
| CA | capsid |
| CCR | chemokine receptor with 2 adjacent cysteines |
| CD | Clusters of differentiation |
| cm | centimetre |
| CRF | circulating recombinant form |
| CXCR | chemokine receptor in which 2 cysteines-C are separted by an amino acid |
| C | Cytosine |

| | |
|---|---|
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| ELISA | enzyme-linked immunosorbent assay |
| Env | envelope |
| Fig | figure |
| Gag | group specific antigen |
| gp | glycoprotein |
| G | Guanine |
| HAART | Highly active antiretroviral therapy |
| HIV | Human immunodeficiency virus |
| HIV-1-M | HIV-1 group M (major) |
| HIV-1-N | HIV-1 group N (non-O, non-M) |
| HIV-1-O | HIV-1 group O (outlier) |
| IL-2 | Interleukin 2 |
| kb | kilobase |
| kV | kiloVolt |
| LTR | long terminal repeat |
| MA | matrix |
| mA | milliamperes |
| µg | microgram |
| µl | microlitre |
| mg | milligram |
| min | minute(s) |
| MIP | macrophage inflammatory protein |
| ml | millilitre |
| mM | millimolar |

| | |
|---|---|
| NC | nucleocapsid |
| Nef | negative factor |
| ng | nanogram |
| NSI | non- syncytium inducing |
| O.D. | optical density |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| Pol | polymerase |
| PR | protease |
| RANTES | regulated upon activation normal T expressed and secreted |
| RNA | ribonucleic acid |
| Rnase | ribonuclease |
| rpm | revolutions per minute |
| RT | reverse transcriptase |
| SDS-PAGE | sodiumdodecyl sulphate polyacrylamide gel electrophoresis |
| sec | second(s) |
| SI | syncytium inducing |
| ssRNA | single stranded RNA |
| SU | surface glycoprotein |
| *Taq* polymerase | *Thermus acquaticus* polymerase |
| Tat | transactivator of transcription |
| $TCID_{50}$ | tissue culture infectious dose 50 |
| TE | Tris/EDTA |
| TM | transmembrane glycoprotein |
| tRNA | transfer RNA |
| T | Thymine |
| V3-loop | variable loop 3 |

| | |
|---|---|
| Vif | viral infectivity factor |
| Vpr | viral protein r |
| Vpu | viral protein u |
| | |
| w/v | weight per volume |
| WB | Western blot |

### 7.4 Amino acids

| | | | | | |
|---|---|---|---|---|---|
| A | Ala | Alanine | M | Met | Methionine |
| C | Cys | Cystine | N | Asn | Asparagine |
| D | Asp | Aspartic acid | P | Pro | Proline |
| E | Glu | glutamic acid | Q | Gln | Glutamine |
| F | Phe | Phenylalanin | R | Arg | Arginine |
| G | Gly | Glycine | S | Ser | Serine |
| H | His | Histidine | T | Thr | Threonine |
| I | Ile | Isoleucine | V | Val | Valine |
| K | Lys | Lysine | W | Trp | Tryptophane |
| L | Leu | Leucine | Y | Tyr | Tyrosine |

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**Publications**

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I hereby confirm that this thesis entitled: characterisation of primary isolates and infectious molecular clones of human immunodeficiency virus type 1 from Cameroon was entirely caried out by me and that all the materials used have been cited accordingly. This work has not been submitted to any other university.

Heidelberg, 5th December 2001

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Sehr geehrte Damen und Herrn,

hiermit bestätige ich, dass die Dissertation von Herr Denis Manga Tebit in fehlerfreiem Englisch geschrieben ist.

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Mit freundlichen Grüßen

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