DETECTION, QUANTIFICATION AND GENETIC ANALYSES OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 2 (HIV-2) INFECTIONS IN THE GAMBIA, WEST AFRICA.

BY

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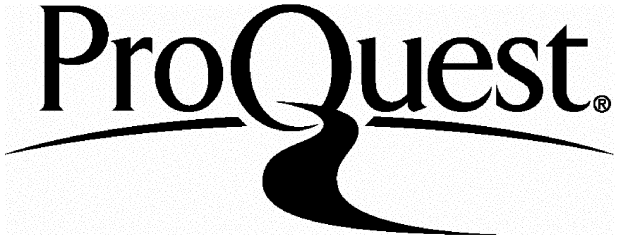

Dedication

This thesis is dedicated to the memory of Sara Partridge, a very dear friend who died recently, lost at sea on the 14th May, 1995.

Acknowledgements.

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Abstract.

Infection with Human Immunodeficiency Virus type 2 (HIV-2) has been investigated in collaborative studies with the MRC unit, Fajara, in The Gambia, West Africa using virological, serological and molecular techniques.

Serum antibody levels of anti-HIV-2 were measured with competitive serological assays using culture-derived viral antigens which were applied in field studies in West Africa. These were subsequently replaced with a recombinant β-galactosidase fusion protein of an immunodominant region of the transmembrane glycoprotein (gp36) of HIV-2. Competitive EIAs were demonstrated to be a specific and sensitive means of detecting anti-HIV-2 and, in most cases, for speciation of HIV-1 and HIV-2 infections and for the detection of related non-human primate lentiviruses. A recombinant HIV-2 p26 antigen was produced as a glutathione-S-transferase fusion protein by PCR amplification and cloning into the pGEX-3X expression vector. These studies yielded a soluble recombinant antigen which was used as an immunogen to raise a polyclonal anti-p26-specific reagent. An assay for the detection of p26 antigens was also developed. Partial nucleotide sequence of the p26 gene of four Gambian HIV-2 strains (CBL.20-23) indicated these to be subtype A strains.

Nested PCR assays were applied to HIV-2 proviral DNA detection using oligonucleotide sequences in the Long Terminal Repeat (LTR), *pol*, and *vpx* regions of the HIV-2 genome. In a cohort of Gambian seropositive individuals, HIV-2 provirus was detected in DNA extracted from PBMCs in 84/86 (97%) of individuals with LTR primers and 39/41 (95%) in a similarly conserved region of *pol* (integrase). LTR sequences and recombinant *Pfu* DNA polymerase, rather than *Taq* polymerase, were applied in a radiometric incorporation assay for quantification of HIV-2 proviral DNA and evaluated by an end-point limiting dilution method. A wide range of proviral DNA levels were found in HIV-2-infected individuals where increased proviral loads, expressed as copies per 10^5 CD4-positive lymphocytes, were strongly associated with CD4 depletion. Nested HIV-1 and HIV-2 PCR primers were also used to identify dual infections.

Amplification and direct sequence analysis of the U3 region of the HIV-2 LTR, from either proviral DNA or viral RNA, indicated a high level of sequence conservation of four *cis*-acting elements *in vivo*. A PCR-based approach to genotyping HIV-2 strains was also developed, capable of differentiating HIV-2 subtype A strains from subtype B or SIV-like viruses based on U3 sequence-specificity. Subtype A viruses were found to predominate in the Gambian samples analysed.

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Chapter 1.

General Introduction.

1.a. The *Retroviridae.*

Retroviruses are extremely diverse in nature. Thin-section electron micrographs have revealed the morphological nature of retroviruses, which have been grouped into A-type, B-type, C-type and D-type particles, a feature which is related to virus assembly (Weiss *et al*, 1985; Gelderblom, 1991). Differences in the assembly process of retroviral components results in fundamentally different viruses. A-type particles are found only as intracellular structures and are non-infectious but may form the precursors of certain B-type viruses. C-type particles exhibit no signs of assembly within the infected cell until budding occurs when the envelope and the immature core develop concurrently. In contrast, B/D type particles pre-assemble as immature cores within the cytoplasm prior to budding. Retrovirus taxonomy, however, is not straightforward and has been based more upon morphological and biological features, including pathogenic properties and host of origin and may also be classified as amphotropic, ecotropic, xenotropic and dual tropic retroviruses. Inclusion as members of the *Oncovirinae*, *Spumavirinae* or *Lentivirinae* is therefore dependent on all or some of these characteristics.

A number of general features are common to all retroviruses, however, and justify their inclusion in the same taxonomic group. Enveloped virions occur as spherical particles approximately 100-140nm in diameter with a buoyant density in caesium chloride of 1.16 g/ml. This compares with core particles which have a buoyant density of 1.21 g/ml in caesium chloride. The virion genome is diploid, comprising two linear positive-sense single-stranded RNA molecules in a ribonuclear complex encapsulated by an icosahedral capsid. The general organisation of the genome is common to all retroviruses, consisting of three structural genes, encoding the core proteins (*gag*), the polymerase enzyme (*pol*), and the envelope (*env)* glycoproteins which encapsulate the virion. Flanked by two Long Terminal Repeat (LTR) sequences the basic structure of retrovirus genomes is always LTR-*gag-pol-env*-LTR. Intact virions consist of 60-70% protein, 2-4% carbohydrate, 1% RNA and a lipid component of 30-40% which is derived from the cell wall of the host as budding and release occurs. HIV-2 is classified as a C-type retrovirus on

morphological grounds belonging to the *Lentivirinae* sub-family. An electron micrograph of HIV virions budding from T-lymphocytes is shown in Figure 1.1.

One of the most characteristic features of retroviruses is possession of a virus-encoded RNA-dependent DNA polymerase (reverse transcriptase) which directs transcription of viral RNA in the mature virion into a proviral DNA intermediate. This is one of the hallmarks of retrovirus activity and allows integration of retroviral genetic information into the DNA of the infected host. Retroviruses may be present as endogenous elements transmitted vertically in the germ line of the infected host, or as exogenous infectious agents. Endogenous retroviruses are mostly defective although may be transiently expressed in host cells (Coffin, 1985), whereas exogenous viruses are usually infectious and often associated with pathology. The diversity of retroviral forms may account for the broad spectrum of pathological disease associated with this group of viruses, including tumour induction and malignancy, immune deficiency and neurological impairments.

1.a.1. Aspects of retroviral taxonomy.

Viruses belonging to the *Oncoviriniae* and the *Lentivirinae* have been identified as important pathogens implicated in causing a range of pathological conditions in humans, although this remains to be demonstrated for members of the *Spumavirinae*.

i. the *Oncovirinae*.

Oncoviruses have been found within all vertebrate species, some groups of insects and reptiles and form the majority of animal retroviruses. Study of members of the *Oncovirinae* has contributed immensely to our understanding of the mechanisms involved in malignant growth, since oncoviruses are typified by their ability to transform cell lines *in vitro* and induce tumour formation *in vivo*. Five groups of oncoviruses have been identified and certain members of each group serve as role models for oncoviruses in the development of tumours and neoplasias.

The *avian leukosis sarcoma* group contains Rous sarcoma virus and avian leukosis virus (ALV), both of which are exogenous viruses and contain oncogenic elements. The ALVs

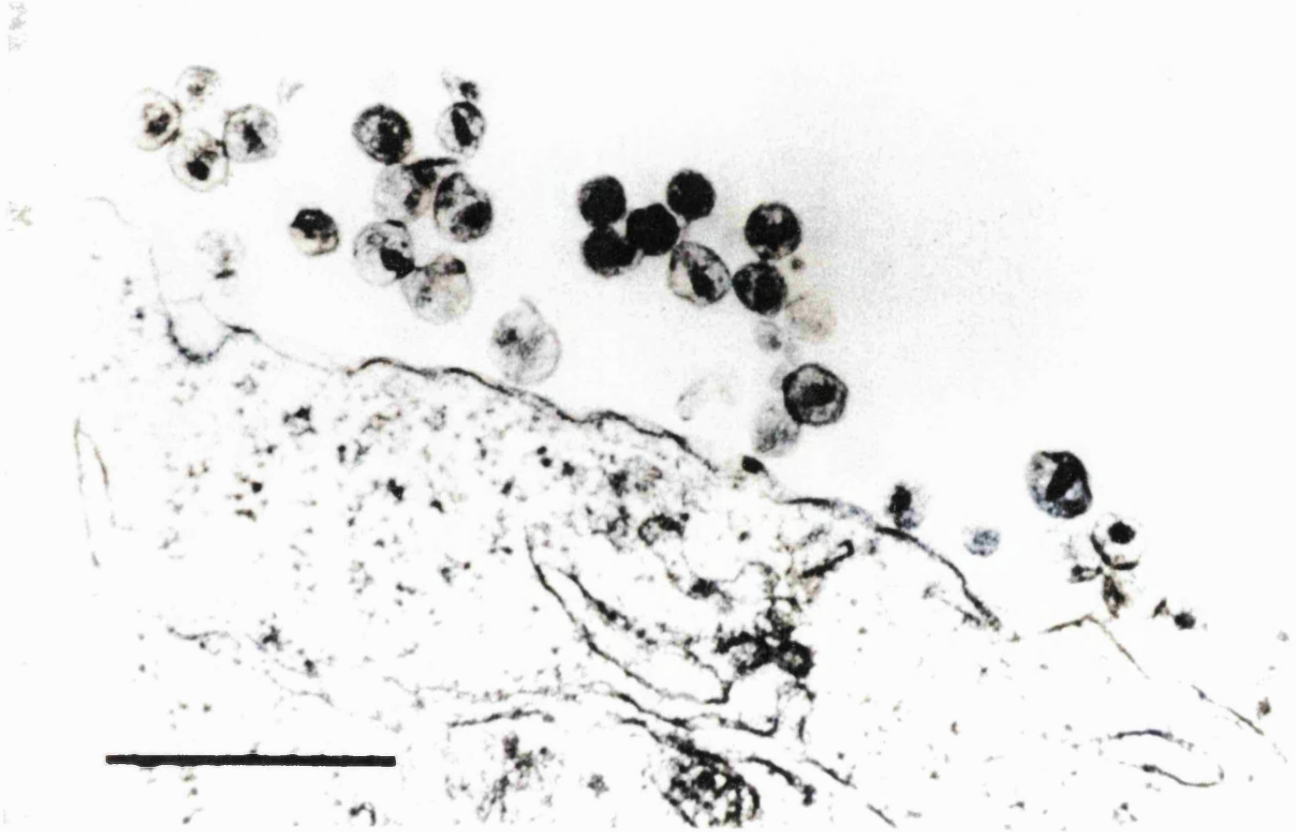


Figure 1.1. Electron micrograph of HIV particles budding from infected cells.
Scale bar = 1 micron (1000 nanometres).

are the major aetiological agents of leukaemia in domestic fowl. The c-*myc* gene is a cellular proto-oncogene which has been implicated in tumour induction in a variety of species. Although it may be activated by both viral and non-viral factors, the integration of ALV proviral sequences within or adjacent to the c-*myc* gene appears to induce expression of *myc* RNA. This appears to be as a direct result of viral promoters within the 3' LTR having an effect on flanking cellular DNA. Although promoter insertion is important in initial activation it is only one step in a very complex process, but serves to exemplify the potential role of retroviruses in carcinogenesis (Weiss *et al*, 1985).

C-type mammalian retroviruses, are also exogenous and cause leukaemias/sarcomas and include murine leukaemia virus (MLV) and feline leukaemia virus (FLV). Interactions between c-*myc* and the FLV provirus have also been linked to T-cell leukaemias (Neil *et al*, 1984; Mullins *et al*, 1984) and in activating T-cell lymphomas with MLV in mice. Lentiviruses, while representing a distinct and separate subgroup, are also morphologically C-type retroviruses.

B-type viruses occur as both exogenous and endogenous forms and insights into the mechanisms of tumour biology have been gained by the study of these RNA tumour-inducing viruses. Mouse mammary tumour virus (MMTV) induces sporadic mammary adenocarcinomas in infected female mice and is perhaps the best studied member of this group (Morris, 1991). The proviral genome is capable of insertion into the host close to *int* genes causing activation of these host proto-oncogenes through transcriptional regulatory elements within the MMTV LTR. Their effects on *int* gene transcription and insertion mutations associated with MMTV have been classified as either enhancer insertions which enhance transcription or promoter insertions which direct the initiation of transcription. Promoter insertions have been identified in the 3' LTR to drive transcription of the mutated *int* gene. Enhancer insertions are more commonly associated with MMTV and are postulated to activate *int* gene transcription through an enhancer in the 5' MMTV LTR. Altered expression of these proto-oncogenes by integration of MMTV LTR sequences has been causally linked with proliferation of tumours in the mammary gland.

D-type particles have only been found in primate species, the most notable example of which is Mason-Pfizer monkey virus (MPMV) which is associated with a type D morphogenesis in mammalian cells. Particle formation resembles A-type retroviruses with a complete intracellular nucleocapsid formed in the infected cell cytoplasm, comprised of a *gag*-precursor which migrates to the plasma membrane prior to release by budding. The *gag* gene of MPMV encodes five polypeptides, a matrix protein (p10), a phosphoprotein (pp16-24), the capsid antigen (p27), a nucleocapsid (p14) and p12. The p12 protein is thought to be critical for MPMV capsid assembly and for the infectivity of mature virions (Sommerfeld *et al*, 1992). This is in contrast to C-type particles which do not contain an equivalent polyprotein precusor and exhibit a different maturation process where assembly and budding occur concurrently at the plasma membrane.

Human T-cell lymphotropic virus/ bovine leukaemia virus (HTLV/BLV) retroviruses resemble C-type viruses in their morphology, particularly lentiviruses in their mode of budding and central nucleocapsid formation, although differ significantly in the appearance of the envelope proteins. The group is important in terms of human disease since HTLV was the first retrovirus to be causally linked with neoplasia in humans. HTLV was independently isolated from individuals suffering from adult T-cell lymphoma/leukaemia in south-west Japan and the Caribbean basin (Poiez *et al*, 1980; Miyoshi *et al*, 1981; Yoshida *et al*, 1982; Reitz *et al*, 1983). HTLV-1 is an exogenous, infectious virus and has also been linked with chronic neurological disorders known as tropical spastic paraparesis and HTLV-associated myelopathy. Transmission occurs via breast-feeding, sexual contact, blood transfusions and the injection of intravenous drugs. A second related virus, HTLV-2, has also been identified which was implicated in two cases of atypical hairy cell leukaemia (Kalyanaraman *et al*, 1982). HTLV-2 shares about 60% sequence homology with HTLV-1, exhibiting extensive serological cross-reactivity. HTLV-2 appears to be epidemiologically distinct from HTLV-1, linked with the use of intravenous drugs (Tedder *et al*, 1984) with a higher prevalence found in southern Europe (Weber *et al*, 1992). HTLV-2 has also been linked with neurodegenerative disorders (Jacobson *et al*, 1993) although direct pathological effects of HTLV-2 in humans remains unclear. An increased prevalence of HTLV-2 has also been found in native American Indians (Levine *et al*, 1993) and in pygmy populations in Africa

(Goubau *et al*, 1993) although this has not been strongly linked with excess morbidity. Simian counterparts similar to HTLV-1 and HTLV-2 have been isolated from several Old World monkeys, termed primate T lymphotropic viruses (Sherman *et al*, 1992). One of the striking properties of HTLV isolates, however, is the relatively high level of conservation of the genome when compared with the wide degree of genetic variation among members of the *Lentivirinae*.

Bovine leukaemia virus (BLV) is also a member of this group of oncoviruses which infects the B cells of cows inducing a B-cell lymphoma following viral replication. These biological properties serve to distinguish BLV, HTLV-1 and HTLV-2 from the lentiviruses as well as having distinct regulatory genes, *tax* and *rex*, at the 3' end of the genome involved in transcriptional regulation.

ii. the *Spumavirinae*.

Spuma (foamy) viruses are exogenous retroviruses with a broad host range in mammalian cells inducing multinucleate giant cell formation, hence the characteristic cytopathic effect of these agents (Neumann-Haefelin *et al*, 1993). Clear links with foamy virus infection in humans and disease still remains to be identified (Weiss, 1988a). Human Foamy Virus (HFV) genomes consist of *gag*, *pol* and *env* genes common to all retroviruses (Maurer and Flugel, 1988) and a region containing the *bel* genes. *Bel 1* is thought to be a *trans*activator of transcription, sharing some homology with the HIV-2 *tat* gene (Rethwilm *et al*, 1991) although the mechanism of transactivation of these viruses is different. Expression of *bel 2* has been associated with increased infectivity (Yu and Lineal, 1993). Cytoplasmic and granular fluorescence of *bel 3* resembles that of HIV-1 *nef* and amino acid homology of *bel 3* with HIV-2 *nef* exists (Maurer and Flugel, 1987). *Bel 3* proteins are present in the cytoplasm of HFV-infected cells although at lower amounts compared to *bel 1* and *bel 2* proteins (Weissenberger and Flugel, 1994).

iii. the *Lentivirinae*.

Equine infectious anaemia virus (EIAV) was first demonstrated to be a transmissible, filterable agent at the beginning of the century (Vallee and Carre, 1904) and in common with all other lentiviruses occurs as a C-type infectious particle. Seven lentiviruses have

now been characterised, including other 'classical' lentivirus infections such as maedi-visna virus (MVV) which was first linked with neurological and degenerative symptoms and progressive lethal pneumonia in sheep (Sigurdsson *et al*, 1957). Caprine arthritis encephalitis virus (CAEV) causes pathological effects in goats. Transmission of these agents is different whereby EIAV and CAEV are both spread by arthropod vectors whereas MVV is transmitted from mother to offspring. More recently, lentiviruses have been linked with lytic infections often resulting in severe immunodeficiency within their respective hosts including bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV), and human immunodeficiency viruses types 1 and 2 (HIV-1, HIV-2). Morphologically and biochemically these are similar to one another, containing a magnesium dependent reverse transcriptase. Non-human lentiviruses, in particular FIV and SIV, have served as models for HIV infection in humans. The immunodeficiency viruses share similar genome organisations and life cycles within the *Lentivirianae,* although perhaps their most striking characteristic is the overall degree of genetic variation, reflected both within and between the different groups of viruses.

1.a.2. Lentivirus infections associated with immunodeficiency.

The association of lentiviruses with severe immunodeficiency is a relatively new phenomenon and the study of immunodeficiency viruses has in recent years become a large area of research, comparable in many respects to the study of the oncoviruses in the field of tumour biology.

i. Feline immunodeficiency virus.

FIV strains are widely found in both feral and domestic felid populations. Comparisons have been made between FIV infection in cats and HIV infection in humans and parallels in the molecular organisation of the genome of these viruses described (Harbour, 1992). T-cell depletion and related immunodeficiency in FIV-infected domestic cats has been documented (Ackley *et al,* 1990a). A CD4 homologue in cats has also been shown to exist for FIV (Ackley *et al,* 1990b). Infection with FIV appears to cause a selective reduction in CD4+ cells, similar to that observed in humans and FIV has been used as a model for studying immunodeficiency induced by lentivirus infection (Novotney *et al,*

1990). Transmission of FIV-infected cells by biting is more important for transmission than sexual contact. A number of non-domestic felid species have also been shown to harbour strains of FIV in different parts of the world. These include free-ranging populations of cheetahs and lions in Africa, and pumas and panthers in North America where geographically separate viruses are also phylogenetically distinct from one another (Olmsted *et al,* 1992). In these free-ranging large cats no pathological or immunological symptoms have been identified. Further serological surveys of free-ranging African and Asian lions have also revealed FIV to be endemic in these populations of large cats with a seroprevalence as high as 90% having been recorded (Brown *et al,* 1994). Genetically, the viruses in the African lions are closely related to each other and distinct from other feline lentiviruses suggesting co-evolution has occurred. Similarly, no apparent link with pathology has been demonstrated.

ii. Bovine immunodeficiency virus.

Bovine immunodeficiency virus (BIV) was first isolated from cattle showing signs of impaired immunity during the early 1970s (Van der Maaten *et al,* 1972), although few studies describing a pathogenic link between BIV infection and disease have been described (Carpenter *et al,* 1992). Experimentally infected cattle, however, exhibit an early transient lymphadenopathy compatible with symptoms observed in other hosts infected with immunodeficiency viruses. However, in spite of some disturbances in lymphocyte and neutrophil functions in the months following infection (Flaming *et al,* 1993), the long term effects of BIV infection remain unknown and the significance of BIV as a pathogen in its natural host remains to be determined.

iii. Simian immunodefiency viruses.

The simian immunodeficiency viruses (SIV) are the closest known relatives of HIV in humans and have received close attention both in terms of the development of putative vaccines for AIDS in humans and as the potential progenitors of HIV-1 and HIV-2 infecting man. The natural hosts for members of the SIV group are Old World African primate species which are genetically diverse and SIV strains have been designated according to species of origin (Franchini and Reitz, 1994). These include the sooty mangabeys (*Cercocebus atys,* SIVsm), mandrills (*Papio sphinx,* SIVmnd), African green

monkeys (*Cercopithecus* spp, SIVagm) and the Sykes' monkeys (*Cercopithecus mitis alboglugaris,* SIVsyk).

The SIVagm group of viruses can be further sub-divided into four virus-groups according to each species from which each virus was isolated. These are the vervet (*Cercopithecus pygerythrus,* SIVagmver), grivet (*Cercopithecus aethiops,* SIVagmgri), tantalus (*Cercopithecus tantalus,* SIVagmtan) and sabaeus (*Cercopithecus sabaeus,* SIVagmsab) monkeys. Asian macaques can also be sub-divided into rhesus macaques (*Macaca mulata,* SIVmac), pigtail macaques (*Macaca nemestrina,* SIVmne), cynomologous macaques (*Macaca fascicularis,* SIVcyn) and stumptail macaques (*Macaca arctoides,* SIVstm). In contrast to natural SIV infection in sooty mangabeys and African green monkeys which does not appear to be linked with pathological effects in the respective hosts, SIV infection in macaques leads to cytopathic killing of target cells, principally those bearing the CD4 antigen, causing an immunodeficiency-like illness similar to AIDS in humans (Hirsch and Johnson, 1994). In particular, this has provided an animal model for the study of pathogenesis of human lentivirus infection.

Lentiviruses have also been isolated from the common chimpanzee (*Pan troglodytes,* SIVcpz) held in captivity in Europe, although these animals originated from Gabon and Zaire (Peeters *et al,* 1989). However, serological screening of feral populations of chimpanzees have not revealed the presence of SIVcpz in their native habitat and interpretations based on the isolation of this virus have been viewed with caution. Numerous species of non-human primates have therefore been identified as being infected with different SIV strains, indicating that a diverse pool of lentiviruses exists in these feral monkey populations.

iv. Isolation of human immunodeficiency virus type 1.

The putative causative agent(s) for the Acquired Immune Deficiency Syndrome (AIDS) in humans was first isolated in Paris, France from a homosexual male suffering from profound immunodeficiency (Barre-Sinnousi *et al,* 1983). This virus was first named lymphadenopathy-associated virus (LAV) because of the clinical presentation of the disease which resulted in severe immunological impairment, in particular of the lymphatic

system. The disease was primarily associated with immunodeficiency whereby AIDS patients became severely immunocompromised, characterised by a range of opportunist infections including viruses, bacteria and other micro-organisms.

It is now widely accepted that infection with HIV is causally linked with AIDS, although other infections may play a role in pathogenesis acting as co-factors in conjunction with HIV. At the time, however, little was known about this novel agent which was subsequently shown to be distinct from HTLV. In contrast to HTLV, it caused a lytic infection and the destruction of the cells it infected although was it was called HTLV-III in the United States (Gallo *et al*, 1984). HTLV-III/LAV was subsequently established in continuous cell lines (Popovic *et al*, 1984) and the expression of viral antigens led to the development of a diagnostic test for virus-specific antibodies in the USA and Europe. Subsequently, the virus was renamed as Human Immunodeficiency Virus (HIV) following international agreement and standardisation of HIV and HTLV gene nomenclature (Gallo *et al*, 1988). HIV was found to be prevalent in homosexual men, intravenous drug users and the recipients of donated blood and blood products, including haemophiliacs (Cheinsong-Popov *et al*, 1984). HIV has now spread worldwide into the wider heterosexual population with at least seven million infections occuring in sub-Saharan Africa alone where it is a major cause of adult mortality (Gregson *et al*, 1994). HIV represents a major global public health problem, there being relatively few countries in the world that remain free of HIV infection with HIV-related disease an important public health issue in other areas of the world, such as Eastern Asia and South America.

v. Identification of human immunodeficiency virus type 2.

During the mid-1980s West African countries were experiencing few cases of HIV infection compared with Central and East Africa. In 1985, Barin and colleagues identified antibodies in the serum of healthy female prostitutes in Senegal, West Africa which reacted strongly in a serological assay incorporating viral antigens derived from SIVmac. In 1986, an AIDS-like illness was diagnosed in two patients, one living in the Cape Verde Islands, and the other from Guinea Bissau, West Africa. Virological and serological investigations proved negative for HIV and subsequent cultivation of peripheral blood mononuclear cells (PBMC's) yielded a retrovirus which was

antigenically related to HIV, but distinct from it (Clavel *et al,* 1986a). Furthermore, this virus was more closely related to SIV than to HIV and became the prototypic strain of HIV-2, designated HIV-2_{ROD}. All previous HIV isolates became known as HIV-1.

Further serological studies identified HIV-2 to be the dominant HIV-type circulating in West Africa (Clavel *et al,* 1986b; Kanki *et al,* 1987). The genetic composition and gene products of the prototypic HIV-2 isolate, HIV-2_{ROD} were also determined (Clavel *et al,* 1986c; Guyader *et al,* 1987) and shown to differ from those of HIV-1 in numerous respects, although there are many similarities in the function of the virion-encoded proteins. Further characterisation confirmed HIV-2 to be both antigenically and molecularly more closely related to strains of SIV than to the HIV-1 group of viruses (Chakrabarti *et al,* 1987; Franchini *et al,* 1987; Kestler *et al,* 1988).

1.a.3. Genomic organisation of HIV-1 and HIV-2.

Molecular cloning and elucidation of the full nucleotide sequence of the genomes of HIV-1 (Wain-Hobson *et al,* 1985; Ratner *et al,* 1985) and HIV-2 (Guyader *et al,* 1987) indicated both to be approximately 9.7kB in length consisting of LTR-*gag-pol-env*-LTR, the typical structure of retroviral genomes. Unlike other C-type retroviruses, however, the HIV-1 genome encodes for further six proteins; *tat* (*trans*-*a*ctivator of *t*ranscription), *rev* (*r*egulator of *e*xpression of *v*irion proteins), *nef* (*ne*gative *f*actor), and *v*iral *p*roteins *vpr*, *vif* and *vpu*. *Vpu* is absent from HIV-2 and SIV isolates although they contain another unique central region gene, *vpx* which is not present in HIV-1 strains. Such a repertoire of accessory genes makes HIV a complex retrovirus and distinguishes it from other animal C-type retroviruses which have a much simpler genetic composition. The organisation of the HIV-2 genome identifying the structural, regulatory and accessory genes and their major gene products is shown in Figure 1.2.

i. Replication and generalised life cycle of HIV.

Following attachment and entry into target cells, two single strands of virion RNA are reverse transcribed into double stranded DNA molecules. This process is mediated by a virus-encoded reverse transcriptase, the first discovery of which was recognised as one

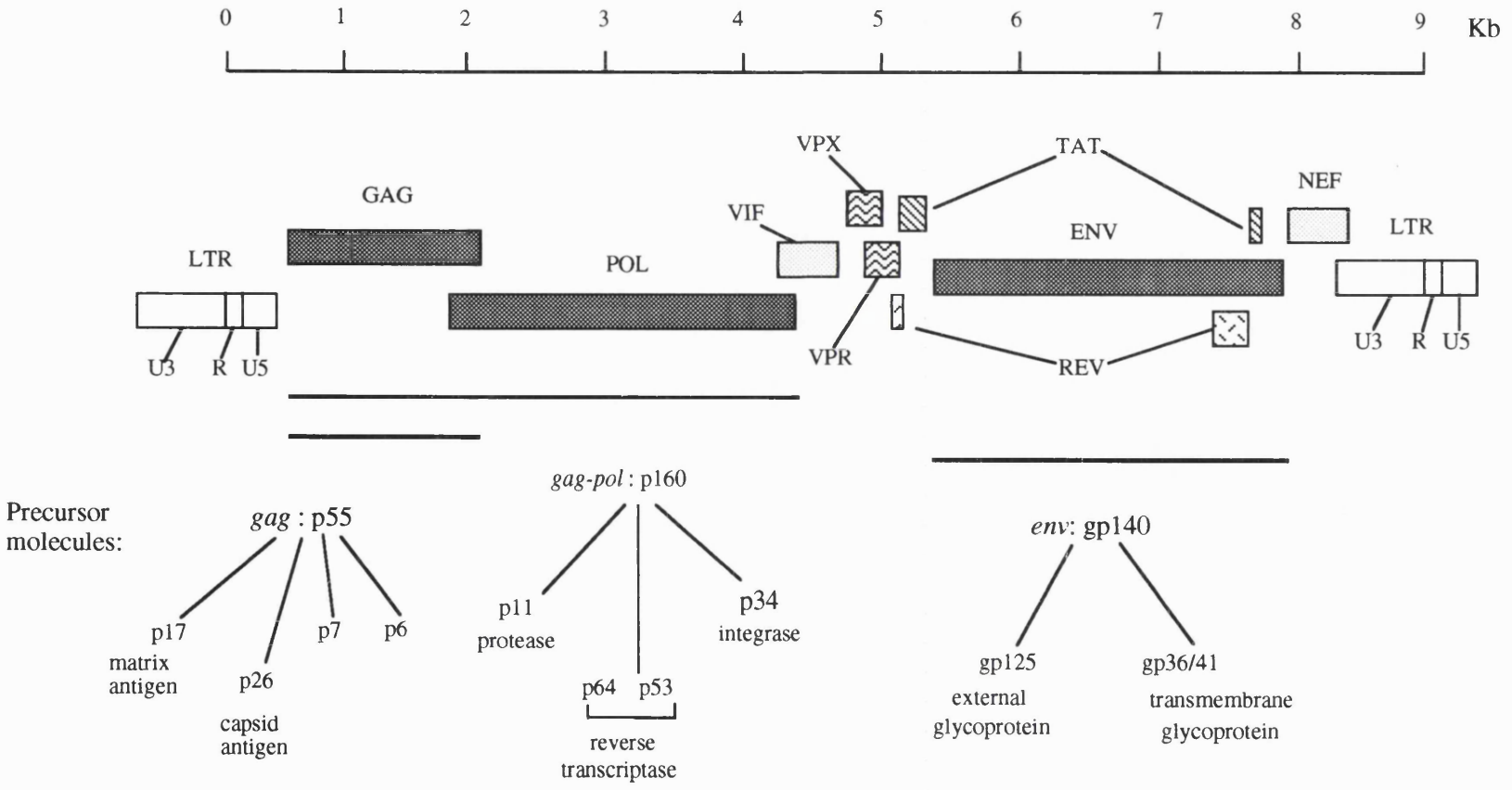


Figure 1.2. Molecular organisation of the HIV-2 genome and virion-encoded proteins.

of the milestones in the field of molecular biology, where the flow of genetic information is reversed from RNA to DNA (Baltimore, 1970; Temin and Mizutani, 1970).

The process of reverse transcription begins by the addition of 100-200 nucleotides from the primer binding site of the tRNA lysine molecule, which is cellular in origin and initaites elongation from the U5 region, to the R region of the LTR at the 5' end of the genome. Ribonuclease H activity digests the RNA part of the RNA-DNA heteroduplex and the newly made DNA copy of R at the 5' end base-pairs with the complementary region of R at the 3' end of the genome. Following conversion from this single strand to double-stranded DNA, integration of proviral DNA into host cellular DNA is mediated by virus-encoded enzymes (integrase and reverse transcriptase) following circularisation into a pre-integration form of the provirus. HIV thereby exhibits both virion RNA and proviral DNA in its life-cycle. The complexities of the life cycle of HIV have been reviewed elsewhere (Bryant and Ratner, 1992) and only a brief account is given here.

Once integrated, the provirus is largely dependent on its host relying on cellular RNA polymerase II for processing of RNA transcripts and protein synthesis on host polyribosomes. Transcription of integrated proviral DNA gives rise to multiple messenger RNA (mRNA) transcripts which encode viral components. The structural proteins are synthesized as a result of the generation of viral mRNA encoding polyprotein precursors. The *gag* gene encodes a polyprotein precursor molecule (Pr55*gag*) and a second polyprotein precursor, Pr160*gag-pol* is also produced such that the two genes overlap by about 240bp. Ribosomal frameshifting along the mRNA during translation is mediated by the short sequence in the overlap which allows expression of the *gag* and *pol* genes with an apparent overproduction of *gag* products. Post-translational cleavage of the p55 *gag* protein complex produces biologically active molecules which assemble into mature and infectious particles.

Genomic RNA also plays an important role in the production of progeny virions not only in interactions with regulatory proteins but also by encoding sequences specific for packaging of the viral genome. Many retroviruses have a packaging sequence, including HIV-1, which is a non-coding region in a highly structured part of the RNA between the

5' splice donor site and the start codon (AUG) of the *gag* gene (Harrison and Lever, 1992). This acts as a signal for encapsulation of full-length genomic RNA and is important for efficient particle formation and the release of infectious virus (Richardson *et al*, 1993). However, there appears to be a lack of this classical packaging signal in this region in HIV-2, suggesting there may be a different mechanism to control this process (Garzino Demo *et al*, 1993).

The sum of these complex processes is the accumulation of HIV structural proteins on the inner surface of the host cytoplasmic membrane where virus assembly and budding are controlled by the interactions between the *gag* proteins, the plasma membrane and virion RNA. Immature viral particles begin to form containing the two single strands of RNA, an incomplete capsid and the envelope glycoproteins which accumulate on the inner surface of the membane. Release of progeny virions, mediated by budding from the infected cell, is the final step in the life-cycle which is summarised in Figure 1.3.

ii. Regulatory genes of HIV-2.

The functions of the principal regulatory genes of HIV-1, *tat, rev* and *nef* have been reviewed elsewhere (Lever *et al,* 1991). The analogous proteins for HIV-2 have not been so comprehensively studied although an analysis of SIV regulatory elements has been instructive in understanding the mechanisms involved in the control of HIV-2 gene expression (Cullen and Garret, 1992).

The HIV-2 LTR is a highly conserved and compressed region which contains multiple regulatory elements (Arya and Gallo, 1988). In common with other retroviral LTR's, these are important in integration of proviral DNA into the host chromosome and in viral gene regulation. Interactions with the LTR, particularly the U3 sequences, may be relevant with regard to the latency of HIV-2 (and HIV-1) since increased viral replication seems to be an important correlate in the progression to disease following HIV infection. Differences in transcriptional enhancers between HIV-1 and HIV-2, including the number of functional NF-κB regulatory elements, associated with the response to activation of resting T cells' have been reported (Arya, 1990; Tong-Starksen *et al*, 1990; Markovitz *et al,* 1990; Markovitz *et al*, 1992). The HIV-2 LTR contains only a single

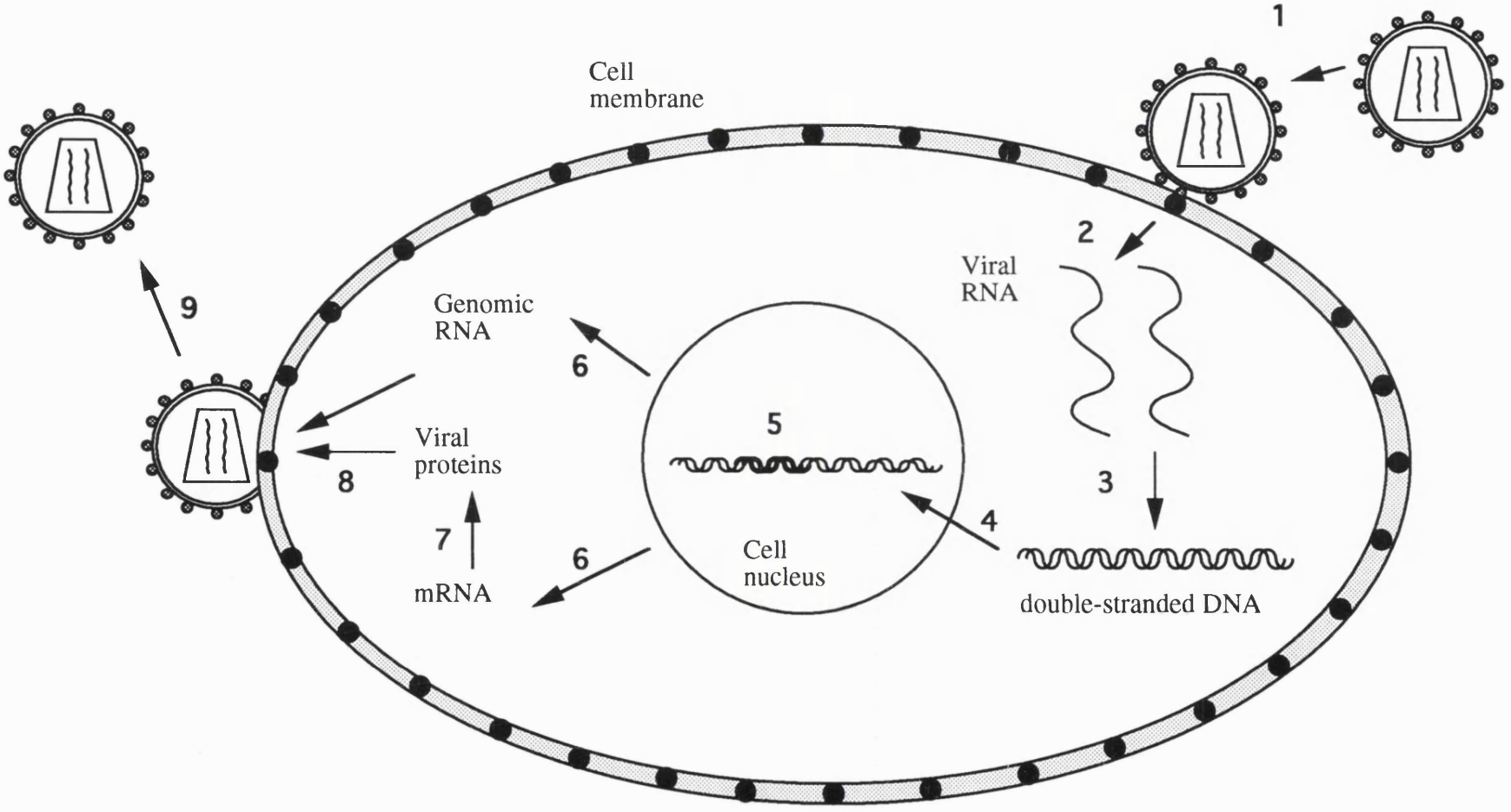


Figure 1.3. Life cycle of HIV. Generalised life and replication cycle of HIV. **Key:** 1. Attachment. 2. Uncoating. 3. Reverse transcription. 4. Integration. 5. Stably integrated provirus. 6. Transcription. 7. Translation. 8. Virus assembly. 9. Budding and release.

functional NF-κB enhancer whereas the HIV-1 LTR codes for two NF-κB elements. Transactivation in HIV-2 is mediated more by specific cellular transcription factors acting through a series of *cis*-acting sequences (Markovitz *et al*, 1992). These observations have been suggested to account, in part, for differences in pathogenicity between HIV-1 and HIV-2 (Markovitz, 1993) although such observations have been made following *in vitro* studies using laboratory isolates. This topic is dealt with in more detail in Chapter 6.

Tat, *rev* and *nef* are expressed early after infection whereas the structural proteins are expressed later in the replication cycle. HIV-2 *tat* proteins are required to produce high levels of viral replication acting in *trans* to increase viral replication, acting through an RNA target sequence the *trans*-activation response or *tat* acceptor region (TAR). The *tat* proteins of HIV-1 and HIV-2 are considered to be broadly similar in structure and function although it has been demonstrated that the *tat* protein of HIV-2 is relatively poor at transactivating the HIV-1 LTR whereas the *tat* protein of HIV-1 is capable of transactivating the HIV-2 LTR (Emerman *et al*, 1987). In HIV-2 and SIV the TAR elements are larger than in HIV-1 and contain three stem-loop secondary structures, two of which are functional and bind *tat* proteins (Emerman *et al*, 1987; Arya and Gallo, 1988, Fenrick *et al*, 1989; Cullen and Garrett, 1992). Differences in the basic domains of HIV-1 and HIV-2 *tat* proteins have also been shown to be important in conferring specificity in binding to the respective TAR structures (Elangovan *et al*, 1992.) Both of the HIV-2 TAR elements appear to be functional *in vivo* and in particular the second exon of *tat* is required for efficient and optimal transactivation of the HIV-2 LTR by binding HIV-2 TAR RNA (Tong-Starksen *et al*, 1993; Rhim and Rice, 1994).

Rev is also considered to be an essential regulatory component acting through its RNA target in the *env* gene, the *Rev* Responsive Element (RRE), to increase the amount of RNA transcripts found in the cytoplasm of HIV-infected cells. The RRE of HIV-2 is conserved across the HIV-2/SIVmac/SIVsm subgroup and coincides with an RNA folding region within the *env* gene immediately 3' to the transmembrane cleavage site (Lewis *et al*, 1990). Analogous to *tat* function, HIV-1 *rev* proteins are fully functional on the HIV-2 RRE whereas HIV-2 *rev* is not active on the HIV-1 RRE sequence failing to

induce viral structural protein expression (Dillon *et al,* 1991). Despite these differences, the mechanism of action of these proteins within the respective replication cycles points to similar functions for regulation of gene expression in HIV-2/SIV and HIV-1.

Nef is a 27kDa myristyolated protein present in HIV-1, HIV-2 and SIV genomes so named as a *ne*gative *f*actor when first identified and in HIV-1 was implicated in binding a region known as a negative regulatory element (NRE) in the U3 region of the LTR. The role of *nef* in HIV-1 has been controversial. Although fewer studies have been performed on HIV-2 *nef*, earlier observations based on studies in SIV have reported its ability to increase replication rather than decrease it (Kestler *et al,* 1991). Determinants within *nef* may therefore contribute to the cytopathy of HIV-2 according to studies with infectious molecular clones and chimaeric viruses (Talbot *et al,* 1993). Studies of *nef* transcripts using molecular clones of the isolate HIV-2_{BEN} established in a human cell line have shown that specific interaction of a balanced amount of *nef* transcripts with the regulatory function of the NF-*k*B element may influence viral replication (Faisst *et al,* 1993). Mutations of *nef* in HIV-2 constructs result in a delay in the production of virus in CEM cells supporting the notion that *nef* plays a positive role, rather than a negative one, in the infection process (Ryan and Peden, 1993).

Although not strictly considered as regulatory genes, but more as accessory genes *vpx, vif, vpr,* in HIV-2 and *vpu* in HIV-1 remain of interest. The *vpu* gene of HIV-1 encodes a protein containing 81 amino acid residues (Cohen *et al,* 1988; Strebel *et al,* 1988). *Vpu* is involved in the maturation of progeny virions and the efficient release of virus particles from infected cells (Terwilliger *et al,* 1989; Klimkait *et al,* 1990). *Vpu* has only been found in HIV-1 isolates with the exception of a virus isolated from chimpanzees (SIVcpz) originating from Gabon and Zaire, Central Africa (Peeters *et al,* 1989). Genetic characterisation of SIVcpz has indicated it to be phylogenetically more closely related to HIV-1 than to any of the other SIVs and possesses a *vpu*-like gene whereas all other SIV's possess a *vpx* (Huet *et al,* 1990).

Vpx appears to be highly conserved between HIV-2 and SIV isolates coding for a 14.5 kDa protein (Kappes *et al,* 1988; Henderson *et al,* 1988; Yu *et al,* 1988). *Vpx* may have

evolved from *vpr* as a result of a duplication of an ancestral *vpr* gene (Tristem *et al,* 1990 and 1992). *Vpx* is thought to enhance viral infectivity and is a virion-associated structural protein. *In vitro* studies of *vpx*-encoded proteins have suggested intracellular transport and packaging of *vpx* is dependent on its interaction with other virus-specific components, in particular the p26 core protein of HIV-2 (Horton *et al,* 1994) which is involved in regulating the incorporation of *vpx* into virions (Wu *et al,* 1994). Independent detection of *vpu* and *vpx* genes or their gene products may serve as independent and type-specific markers for HIV-1 and HIV-2 infections.

Vif (*v*irion *i*nfectivity *f*actor) of HIV-2, like its HIV-1 counterpart, is membrane-associated and *vif* proteins are found localised in the cytoplasm of infected cells. *Vif* is required for cell-free transmission of HIV-2 virions *in vitro* (Michaels *et al,* 1993).

iii. Role of the CD4 molecule.

The attachment of enveloped viruses to specific cell surface receptors is one of the primary events in virus infection and the HIV envelope is thought to be one of the principal determinants of infectivity. At least five different retroviral cellular receptors have been determined and this complex topic has been extensively reviewed elsewhere (Weiss, 1993). One of these is the CD4 antigen, a 60 kDa glycoprotein, identified as a receptor for HIV soon after its discovery (Dalgliesh *et al,* 1984; Klatzmann *et al,* 1984). CD4 interacts with specific epitopes on the external envelope glycoprotein (EGP) of HIV, which for HIV-1 is a heavily glycosylated 120 kDa protein (gp120). For HIV-2 the EGP has been identified as a 125 kDa (gp125) protein (Rey *et al,* 1989a).

Epitope mapping has demonstrated the CD4 molecule to be the major cellular receptor for HIV-1, HIV-2 and SIV (Sattentau *et al,* 1988; Weiss, 1993) and plays a major role in HIV pathogenesis. Once infection has been established CD4-positive (CD4+) cells may act as a reservoir for HIV (Schnittman, 1989) and the depletion of CD4+ cells from the peripheral circulation is reflected in progression to symptomatic disease. The CD4 molecule has been studied with respect to HIV-1, HIV-2 and SIV in both CD4+ and CD4-negative (CD4-) cell lines (Werner *et al,* 1990). HIV-2 virions may exhibit reduced levels of binding to CD4+ cells under the same conditions compared with HIV-1

(Looney *et al,* 1990). Certain CD4-negative cell lines such as the Daudi B-cell line and rhabdomyosarcoma cells are resistant to infection with HIV-1_{IIIB} although are susceptible to infection with a variant of HIV-2_{ROD}, LAV-2B suggesting an alternative receptor may be involved in the entry of some HIV-2 strains (Clapham *et al,* 1992). HIV-2 has also been described as having a lower affinity for soluble CD4 compared with HIV-1 strains (Moore, 1990). Direct interaction between HIV-2 gp105 and CD4, crucial to viral entry and infectivity has also been demonstrated (Steffy and Wong-Staal, 1993).

Cells other than CD4+ T lymphocytes also bear the CD4 antigen and are capable of supporting HIV replication, including cells of the monocyte and macrophage lineages (Gendelman *et al,* 1989). Macrophages play an important role in AIDS pathogenesis, involved in antigen presentation and cytokine release, activation of other cells of the immune system and as a reservoir for HIV-1 (Collman *et al,* 1990; Cheng-Mayer *et al,* 1990). In HIV-1 infection, other cells of the macrophage/monocyte lineage also bearing CD4 may become infected including alveolar macrophages in the lung and microglial cells in the brain (Jordan *et al,* 1991). Dendritic cells of the blood and lymph nodes and Langerhans cells in the skin also bear CD4 and can replicate HIV (Knight *et al,* 1990; Langhoff *et al,* 1991). However, other cell types including certain endothelial, epithelial and certain types of neuronal cells (astroglial and oligodendroglial cells) are CD4-negative but may be infected with HIV *in vitro* although this has not been shown *in vivo.*

The CD4 molecule therefore plays an important role in AIDS pathogenesis mediating entry of HIV into a wide range of cell types including those of the central nervous system and depletion of CD4+ T lymphocytes remains an important marker in the development of clinical disease.

1.b. Natural history of HIV-2.

Acute or primary infection with HIV-1 is accompanied by high levels of virus detectable in the blood and may be accompanied by transient symptomatic disease in a significant proportion of individuals (Niu *et al,* 1993). Symptomatic primary infection has also been documented for HIV-2 (Besnier *et al,* 1990). HIV-1 and HIV-2 have tropism for both macrophages and lymphocytes following primary infection (Valentin *et al,* 1994).

Infectious virus initially cleared from the peripheral circulation is accompanied by the appearance of cytotoxic T lymphocytes (CTLs) and this early viraemic phase subsides to lower levels although the immune system fails to clear completely the virus. Seroconversion occurs with the production of a broad spectrum of virus-specific antibodies, including neutralising antibodies. An asymptomatic period then occurs representing a persistent state during which virus may be released from virus-infected cells periodically, although generally not reaching the levels first observed immediately following primary infection. During the course of infection, two phenotypically distinct forms of HIV may occur with differing biological properties *in vitro* which are either syncitium inducing (SI) viruses or non-syncitia inducing (NSI) viruses. A positive culture in MT-2 lymphocytes using primary patient material as the source of virus correlates with a lower CD4 count in HIV-1 infection and represents a marker for progression to HIV-1 disease (Karlsson *et al*, 1994), although a comparable system for HIV-2-infected individuals has yet to be evaluated. The length of the asymptomatic period varies considerably in HIV-1 infection and some individuals have remained well during this period for up to 15 years (Levy, 1993). However, a reduction in the CD4 count for the majority of HIV-infected individuals is accompanied by the appearance of a range of clinical symptoms and opportunist infections characterising AIDS.

Early studies conducted on HIV-2-infected prostitutes in Senegal suggested that HIV-2 was not associated with an AIDS-like illness since relatively little disease-association could be found in these women (Barin *et al*, 1985; Kanki *et al*, 1987). These preliminary investigations, coupled with some anecdotal observations (Ancelle *et al*, 1987; Dufoort *et al*, 1988) led to the suggestion that HIV-2 infection was associated with a long incubation period or asymptomatic state. This added to speculation that HIV-2 infection was clinically distinct from HIV-1 and that the pathogenic effects of HIV-2 were less than HIV-1, although these were still apparent (Marlink *et al*, 1988; Romieu *et al*, 1990). Portuguese patients have been retrospectively diagnosed with AIDS-like symptoms as far back as 1978 (Saimot *et al*, 1987) and archival sera from West Africa (the Ivory Coast, Gabon and Nigeria) collected in 1966 and 1967 (Kawamura *et al*, 1989) were also reported to contain HIV-2 antibody. HIV-2 is therefore likely to have been present in West Africa since at least this time though few cases of HIV-2 AIDS had been

diagnosed. These reports, however, had to be balanced with others describing a clear association between infection with HIV-2, AIDS-related disease and severe immunodeficiency (Clavel *et al,* 1986a; Brun-Vezinet *et al,* 1987; Albert *et al,* 1989) where the clinical course of advanced HIV-2 infection and AIDS in hospitalised patients was indistinguishable from HIV-1 (Mabey *et al,* 1988; DeCock *et al,* 1990a).

1.b.1. Epidemiological aspects of HIV-2 infection.

HIV-2 infection is endemic in West Africa (DeCock and Brun-Vezinet, 1989), in particular Guinea Bissau, Senegal, The Gambia, Ghana and the Ivory Coast. The relative geographical location of these countries is shown in Figure 1.4. The prevalence of HIV-2 is highest in Guinea Bissau and serological studies have indicated around 10% of the general population to be infected in community-based studies in the capital city of Bissau (Poulsen *et al,* 1989; Naucler *et al,* 1991) and in a rural village in the northern part of the country (Wilkins *et al,* 1993; Ricard *et al,* 1994), compared with 1-2% in most other parts of West Africa. The prevalence of HIV-2 infection is also higher in individuals over the age of 25 who are of Guinea-Bissan nationality (Abbott *et al,* 1994). The age-acquisition of HIV-2 appears to rise more slowly than for HIV-1 during the most sexually active years of life, reaching a peak in 50-55 year olds further suggesting that HIV-2 infection may be associated with a prolonged asymptomatic period before symptomatic disease becomes apparent. Social, ethnic and demographic factors play a major role in the transmission and epidemiology of HIV-2 in West Africa (Wilkins *et al,* 1993).

i. Transmission of HIV-2.

Transmission routes for HIV-2 infection and the risk factors linked to these are similar to those described for HIV-1. Sexual activity has also been strongly linked with the spread of HIV-2 in West Africa. The migration of prostitutes across the region, both to and from areas of high endemicity, has played a major role in transmission (Wilkins *et al,* 1991a; Pickering *et al,* 1992). Movement of males from their homes is reflected in the disproportionate number of females often found in communities in Africa generally, considered to have a significant effect on sexual behaviour and the spread of HIV-1 in

Figure 1.4. Countries of West Africa: the geographical region of HIV-2 endemicity.

East and Central Africa (Larson, 1989). In studies conducted in The Gambia it was demonstrated that over 25% of prostitutes were infected with HIV-2 and that there was also a significant link between infection and the incidence of genital ulcer disease (Pepin *et al*, 1991a). Other sexually transmitted diseases are therefore strongly linked with transmission of HIV-2. Comparisons of the transmission rate between HIV-1 and HIV-2 in Senegal has indicated that the rate of male to female transmission of HIV-2 may be less in comparison with HIV-1 (Donnelly *et al*, 1993).

Transfusion-acquired HIV-2 infection has been reported (Poulsen *et al*, 1989), although the number of these cases may be limited (Wilkins *et al*, 1991a) and the introduction of screening of blood donations in recent years has greatly reduced the chance of being transfused with blood from an HIV-2-infected donor. Blood tranfusions received after 1987 not resulting in any seroconversions in a more recent study also point to the reduced role of this route with the use of effective screening strategies (Poulsen *et al*, 1993).

Vertical transmission has also been documented for HIV-2 (Matheron *et al*, 1990; Morgan *et al*, 1990) although the limited data on this topic suggests that this is unusual for HIV-2, if not rare (Poulsen *et al*, 1992; Andreasson *et al*, 1993). As with HIV-1, however, perinatal rather than intrauterine transmission is believed to occur although little is known about the outcome and survival of off-spring infected with HIV-2 at birth.

ii. Spread of HIV-2 outside of West Africa.

During the mid to late 1980s, the HIV-2 epidemic appeared to be exclusively confined to West Africa with only sporadic cases of HIV-2 infection reported in Central Africa (Georges *et al*, 1988). However, HIV-2 could be linked with countries which had strong colonial links with West Africa and in particular with Guinea Bissau. In other parts of Africa, only Angola and Mozambique have a relatively high prevalence of HIV-2 infection as a result of military connections with Portuguese West Africa. While HIV-2 infection is still largely confined to the countries of West Africa, HIV-2 is present in a number of countries outside of West Africa, mainly in Europe.

HIV-2 in Europe was first identified in several countries including Sweden (Biberfeld *et al*, 1986), Germany (Werner *et al*, 1987), Portugal and France (Saimot *et al*, 1987; Brun-Vezinet *et al*, 1987; Brucker *et al*, 1987). Past colonial links between Europe and West Africa have led to geographical pathways facilitating the spread of HIV-2 between the two continents. Based upon a geographical-historical division of West Africa, Smallman-Raynor and Cliff, (1992) identified the spread of HIV-2 out of West Africa from three source regions. The first and most significant is Portuguese West Africa, where the link between Guinea Bissau and Portugal accounts for almost one third of HIV-2 infections in Europe. An additional intra-European link also exists between Switzerland and Portugal. A second node has been identified focussing upon the countries of French West Africa, principally Senegal, Mali and the Ivory Coast whereby France is the main receptor although cases are widely distributed throughout Europe. The third diffusion node centres on British West Africa, especially The Gambia and Ghana. In addition to the Portugal-Guinea Bissau link, prominent ties between Germany and Sierra-Leone, the Netherlands and Guinea Bissau and Switzerland and the Ivory Coast are evident.

HIV-2 has been identified in the United States (Ayanian *et al*, 1989) although the cases to date are few. In contrast, the prevalence of HIV-2 in Brazil is increasing from early case reports (Cortes *et al*, 1989), where both HIV-1 and HIV-2 have been identified (Pieniazek *et al*, 1991). HIV-2 is also present in India (Rubsamen-Waigmen *et al*, 1991), and both HIV-1 and HIV-2 infections have been identified in high-risk populations, principally prostitutes, in Bombay (Pfutzner *et al*, 1992) and in the southern cities of India (Babu *et al*, 1993). The relatively recent co-introduction of both HIV-1 and HIV-2 into India appears to have resulted in a comparatively high number of dually-infected individuals as demonstrated by DNA sequence analysis (Grez *et al*, 1994). Kaposi's sarcoma has been documented in an HIV-1 and HIV-2-infected Indian woman (Rubsamen-Waigmann *et al*, 1993).

iii. HIV-1 in West Africa.

West Africa has experienced a much lower prevalence of HIV-1 infection and associated AIDS cases compared to Central and East Africa. However, the Ivory Coast has

reported a much higher prevalence of AIDS cases as a consequence of infection with HIV-1 and HIV-2 (Odehouri *et al*, 1989; DeCock *et al*, 1990a). In 1990, up to 40% of adult deaths in Abidjan, the capital city of the Ivory Coast, were attributed to HIV infection and AIDS making it the largest single cause of death in this region (DeCock *et al*, 1990b). Perhaps due to its geographical location, the Ivory Coast has experienced the phenomenon of both viruses co-circulating in the same population.

While very few cases of HIV-1 infection existed in The Gambia in 1987, HIV-1 has spread into this country in recent years (Wilkins *et al*, 1991b) and current seroprevalence data indicates it now to be approximately 0.7% in the general population (Dr. H. Whittle, MRC Fajara). The prevalence of HIV-2 in The Gambia has remained stable at 1.6-1.8% for several years. The phenomenon of both viruses circulating in the same population is also occurring elsewhere in West Africa. In Ghana, HIV-1 is now the dominant type of HIV, where previously mainly HIV-2 was present and has been accompanied by an increase in the number of individuals dually serologically reactive for both viruses (Hishida *et al*, 1994). Guinea Bissau, however, still seems relatively free of HIV-1 infection with a high level of HIV-2 infection persisting in the general population.

1.b.2. Pathogenicity of HIV-2 in humans.

One of the fundamental and most controversial questions still regarding HIV-2 is that of pathogenicity. Both population-based clinical studies of the effects of HIV-2 on the immune system and *in vitro* characterisation of the biological properties of HIV-2 strains have enabled a wider view of HIV-2 to be gained.

i. Immunological studies.

Although differences in the natural history between HIV-1 and HIV-2 have been documented suggesting reduced pathogenic properties for HIV-2, clinical and immunological studies have highlighted the potential for HIV-2 to cause severe immunodeficiency. Community-based studies in Guinea Bissau have shown HIV-2-infected individuals to have significant immunosuppression with reduced total numbers of CD4+ T lymphocytes compared to matched controls (Lisse *et al*, 1990). Studies conducted in other parts of West Africa, where HIV-1 is also present, have compared

both HIV-1 and HIV-2-infected individuals in the same population (Kerstens *et al*, 1992), including a cross-sectional study of prostitutes in The Gambia (Pepin *et al*, 1991b). Measurement of CD4+ and CD8+ T lymphocyte subsets, neopterin and β-2 microglobulin levels have been applied to HIV-2 infection and related to the clinical stage of disease (Whittle *et al*, 1992; Whittle *et al*, 1993). While HIV-2 clearly damages the immune system and is immunosupressive during the asymptomatic phase, the extent to which this occurs is less compared with HIV-1 infection. However, a clear association between a low CD4 count, HIV-2 seropositivity and the clinical manifestation of disease resulting in high mortality in hospitalised patients has supported the view that HIV-2 is clearly pathogenic in humans (Mabey *et al*, 1988; Naucler *et al*, 1991; Naucler *et al*, 1992; Poulsen *et al*, 1993).

ii. *In Vitro* biological properties of HIV-2 strains.

Following the initial isolation of HIV-2/LAV-2_{ROD} (Clavel *et al*, 1986a), the genome was cloned and the full nucleotide sequence determined (Guyader *et al*, 1987) and has served as a reference point or prototype strain for comparison with other HIV-2 strains. As more isolates of HIV-2 were obtained and their genomes sequenced, so it became clear that the genetic diversity within the HIV-2 group was at least as large as that observed with HIV-1 strains at the time (Zagury *et al*, 1988). Isolates were obtained from individuals from different countries within West Africa, mostly from patients with symptomatic disease. Characterisation of each of these has revealed a range of geographically, biological and genetically diverse viruses. These include the first HIV-2 isolate, HIV-2_{ROD} from an AIDS patient on the Cape Verde Islands, HIV-2_{NIHZ} from Guinea Bissau (Zagury *et al*, 1988), HIV-$2_{SBL6669/ISY}$ from The Gambia (Albert *et al*,1987; Franchini *et al*, 1989), HIV-2_{ST} from Senegal (Kong *et al*, 1988), HIV-2_{BEN} which was obtained from a German AIDS patient thought to have been infected in Mali (Kirchoff *et al*, 1990) and HIV-2_{GH-1} from Ghana (Hasegawa *et al*, 1989). HIV-2_{D194} was obtained from a Gambian patient with neurological symptoms related to severe immunodeficiency (Kuhnel *et al*, 1989). A highly divergent strain, HIV-$2_{D205/ALT}$ was also obtained from a Ghanain patient (Kuhnel *et al*, 1989; Dietrich *et al*, 1989). The complete nucleotide sequence of these eight strains therefore represented a database to which subsequent strains could be compared. Although there were only eight, they were

from diverse geographical areas across West Africa. The biological heterogeneity of HIV-2 strains comparing their *in vitro* biological and phenotypic properties has been described, including the HIV-2_{UC} strains from the Ivory Coast (Castro *et al*, 1990), the HIV-2_{CBL} series of isolates from The Gambia (Schulz *et al*, 1990) and the HIV-2_{CAM} isolates from Guinea Bissau (Tristem *et al*, 1989). These studies have demonstrated marked differences in the pathogenicity of HIV-2 strains in culture systems and cell lines.

It was subsequently proposed that the HIV-2 group of viruses can be phylogenetically divided into at least two subtypes. Prototypic HIV-2 strains (subtype A) represented the bulk of strains compared with others such as HIV-$2_{D205/ALT}$, HIV-2_{GH-2} (Kawamura *et al*, 1992) and HIV-2_{UC-I} which formed a second group (subtype B). Most subtype A viruses exhibit cytopathic killing of CD4+ cells in cell culture, although differing phenotypes have been identified. The HIV-2_{ST} isolate has a subtype A genotype but its phenotype is characterised by attenuated virulence and is non-cytopathic, non-fusogenic for CD4-bearing target cells. Alterations in the length of the cytoplasmic domain of the HIV-2 transmembrane glycoprotein may modulate fusogenicity and pathogenicity of this isolate (Kumar *et al*, 1990; Mulligan *et al*, 1992).

HIV-2_{UC-1} was isolated from a patient in the Ivory Coast with neurological symptoms although this isolate grew to high levels with no apparent cytopathic effects in the CD4+ cell lines in which it was cultured and antigen expression on the surface of CD4-bearing cells remained normal (Evans *et al*, 1988a). HIV-2_{UC-1} also has a divergent genotype, clustering genetically with subtype B HIV-2 strains (Barnett *et al*, 1993). Such studies illustrate that HIV-2 strains form a heterogenous group of viruses not only in their genetic composition but also in their biological and growth properties and that some of these are non-cytopathic and associated with attenuated virulence. Similarly, HIV-2_{ALI} is an NSI isolate which does not infect most cell lines although this virus replicates to high titres in fresh PBMCs without causing syncitia formation and was originally isolated from a patient with AIDS-related illness (ARC) from Guinea Bissau (Azevedo Pereira *et al*, 1991). Another newly identified isolate is HIV-2_{HOM}, isolated from an asymptomatic Senegalese individual which is reported to be of reduced virulence (Nick *et al*, 1993), similar to HIV-2_{ST}.

In comparison, the isolate HIV-2_{EHO} is highly virulent and highly infectious mediating cytopathic cell killing as a consequence of apoptosis, requiring cell-surface interactions of envelope glycoproteins (Rey *et al*, 1989b). Genetically, however, HIV-2_{EHO} is reported to be most closely related to HIV-2_{D205} and HIV-2_{UC-1} (subtype B) and is also unusual in that its phenotype is NSI but is recognised to be a highly infectious virus and is expressed to a very high level in CEM cells and CD4+ cells from healthy individuals (Rey-Cuille *et al*, 1994). These studies of phenotypic properties of HIV-2 strains taken together with the broad genetic variation indicate that there is a diverse spectrum of HIV-2 strains infecting humans.

iii. Relationship with non-human primate lentiviruses.

Following the initial characterisation of HIV-2_{ROD}, it was clear that HIV-2 was more closely related to certain strains of SIV than to HIV-1 (Clavel *et al*, 1986a). It was therefore possible that HIV-2 infection in humans was the result of horizontal transmission of simian lentiviruses from non-human primates (Smith *et al*, 1988; Doolittle, 1989). Possible candidates for this were SIVagm from African green monkeys (Kanki *et al*, 1986) although in terms of sequence homology, the sooty mangabey virus (SIVsm) was more similar to HIV-2 and provided a more likely candidate (Hirsch *et al*, 1989a; Marx *et al*, 1991). Sooty mangabey monkeys are indigenous to West Africa with approximately 10% being infected with SIVsm. Studies of Asian macaques have revealed that there are no similar SIVs present in feral populations of these monkeys (Hirsch and Johnson, 1994) with SIVmac probably having arisen from cross-species transmission from a mangabey to a macaque in captivity.

Until relatively recently, virological studies of HIV-2 in humans have mostly concentrated on the characterisation of viral isolates which have principally, although not exclusively, been obtained from individuals with an AIDS-like or 'slim disease' condition in urban areas. Though sequence homology of SIVsm with many HIV-2 strains had therefore suggested this may be the progenitor for HIV-2, no such virus had been identified directly in humans. Investigation of HIV-2 infection in a rural part of Liberia, West Africa demonstrated humans living in rural villages to be infected with highly divergent strains compared to those previously described for HIV-2, mostly isolated

from AIDS patients in the urban areas (Gao *et al*, 1992). Detailed analysis of the genomic sequence of one of these strains, HIV-2_{F0784}, has provided the strongest evidence yet that HIV-2 infection in humans had arisen from a cross-species transmission of a simian lentivirus to humans. Genetically, this virus is virtually identical to the SIVsm viruses previously isolated from sooty mangabey monkeys. SIVsm infection in sooty mangabeys does not appear to be pathogenic and the possibility exists that pathogenic HIV-2 strains in humans may have arisen from non-pathogenic SIVs transmitted horizontally from their natural host.

The wide genetic diversity and broad spectrum of biological diversity of HIV-2 strains may account for some of the differences in clinical disease observed in HIV-2-infected individuals. The ability to detect the widest range of HIV-2 strains, including SIV-like viruses was therefore an important component in the virological studies of HIV-2 infection described in this thesis.

1.c. Collaborative studies with The Gambia.

Population-based studies of HIV-2 infection requires close links with West Africa. The Department of Virology, UCLMS has held collaborations with colleagues at the MRC Unit, Fajara, in The Gambia since 1987, soon after the first descriptions of HIV-2 and its association with West Africa. Situated in the southern Sahel region of West Africa, The Gambia is one of the smallest (10 400km^2) and most densely populated countries (60 persons/km^2) in Africa and is surrounded by southern Senegal. The country is essentially defined by the river Gambia with the population evenly dispersed throughout the country in about 500 villages. HIV-2 has been endemic in The Gambia for a relatively short period of time as available data indicate that infection was low before 1985.

The application and supply of sensitive and specific serological assays for the detection of anti-HIV-2 were important in the establishment and continuation of clinical and virological studies. Subsequent development of polymerase chain reaction-based assays and methodologies suitable for testing clinical specimens have been applied to studies aimed at furthering our understanding of the evolution of disease in HIV-2-infected individuals. The transfer of technologies to The Gambia has therefore been an integral part of collaborative studies and devising assays which perform equally well in Fajara and London, where applicable, have been an important component of the work described in this thesis. An integrated, collaborative approach to research into HIV-2 infection in West Africa has been central to the aims of these studies.

The MRC laboratories and The Gambia river are shown in Figures 1.5. and 1.5.b.

Figure 1.5.a. The MRC laboratories, The Gambia.

Figure 1.5.b. The Gambia river.

Chapter 2.

Materials and Methods.

The methodologies and assay protocols described are those performed under optimal conditions. Where assays have been developed empirically, the experiments performed to determine these conditions are described in the appropriate results section.

2.a. Serological studies.

The serological studies performed describe the measurement of HIV-2-specific antibody (anti-HIV-2) by competitive EIA using two assays. The first is an existing assay, utilising viral antigens (VC-EIA) originating from the *in vitro* propagation of HIV-2 strains and the second which has been developed by incorporating a genetically engineered recombinant protein (RC-EIA). Their performance is also compared with that of a number of commercially available assays.

2.a.1. Competitive EIA using culture-derived virus (VC-EIA).

i. Virus culture.

Virus stocks were maintained in a variety of continuous T-cell lines adapted for the propagation of HIV-1, HIV-2 and SIV strains. All such manipulations were performed in the category 3 containment laboratory within the Department of Virology, UCLMS, according to current safety practices. Disposable plasticware was used for all category 3 manipulations and all waste material was disposed of by autoclaving. Three uninfected cell lines capable of supporting the *in vitro* replication of HIV were used. These were C8166, CEM and J Jhan cells, the last of which had been derived clonally from the mature T-lymphocyte Jurkat cell line. All isolates and uninfected cells were kept in long term storage in liquid nitrogen in Nunc cryotubes (Gibco-BRL Ltd) containing 1ml of freezing medium (Appendix 1). Following retrieval from liquid nitrogen, cells were thawed quickly in a 37°C water bath, washed with 20ml of sterile PBS and pelleted by centrifugation (150 g, 10 minutes) in sealed buckets in a CR3000 bench centrifuge (Jouan Ltd) at 4°C.

CBL-20 was propagated in complete RPMI cell culture medium (Appendix 1) in 175cm^2 tissue culture flasks and incubated at 37°C in an atmosphere containing 5% CO_2 (GA2 CO_2 incubator, Leec Ltd). A mixture of C8166 and CEM cells was found to be the most reliable combination for sustained and continual release of virus although J Jhan cells were also routinely used. Uninfected cells were mixed with infected cells at a ratio of 1:3 every 3-5 days and cultures monitored by light microscopy for evidence of cytopathic effect.

ii. Immunofluoresence.

Cultures were monitored for expression of viral antigens by immunofluorescence. Aliquots (3-5mls) of cells infected with HIV-1, HIV-2, or SIV isolates were pelleted by low speed centrifugation (150g) in universal containers, contained within sealed buckets in a bench centrifuge (Jouan Ltd). Supernatant fluid was removed, the pellet resuspended in PBS and several drops of the suspension layered onto teflon-coated multiwell fluorescent microscope slides (Henley Ltd.) These were air-dried in a class 1 safety cabinet, fixed in cold acetone for 10 minutes and exported from the category 3 laboratory. Slides were stored at -20°C if not required immediately. Sera used to determine the relative degree of expression of cell lines were from HIV-2-infected individuals (BM and DJ) or an HIV-1-infected individual (VB), diluted 1/100 in PBS and absorbed against uninfected C8166 cells at 37°C for 1 hour. These were reacted with virus-infected and uninfected preparations for 3 hours at room temperature in a moist chamber, and washed three times with PBS. A sheep anti-human FITC conjugate (Dako Ltd) was incubated at a dilution of 1/1000 for 2 hours and slides viewed in ultra-violet light with an appropriate filter for fluorescent staining.

iii. Preparation of VC-EIA solid phase.

A competitive EIA for the detection of HIV-2 antibody using culture-derived antigen preparations had been previously established (Tedder *et al*, 1988), as outlined in Figure 2.1. Antigen used in the competitive EIA (VC-EIA) was prepared from the culture of CBL-20 as described in Section 2.a.1.i. Supernatant fluid was harvested from culture flasks and inactivated using fresh β-propiolactone (Sigma Ltd), added to a final concentration of 0.25% at 4°C for at least 2 hours. Inactivation, mediated by acid

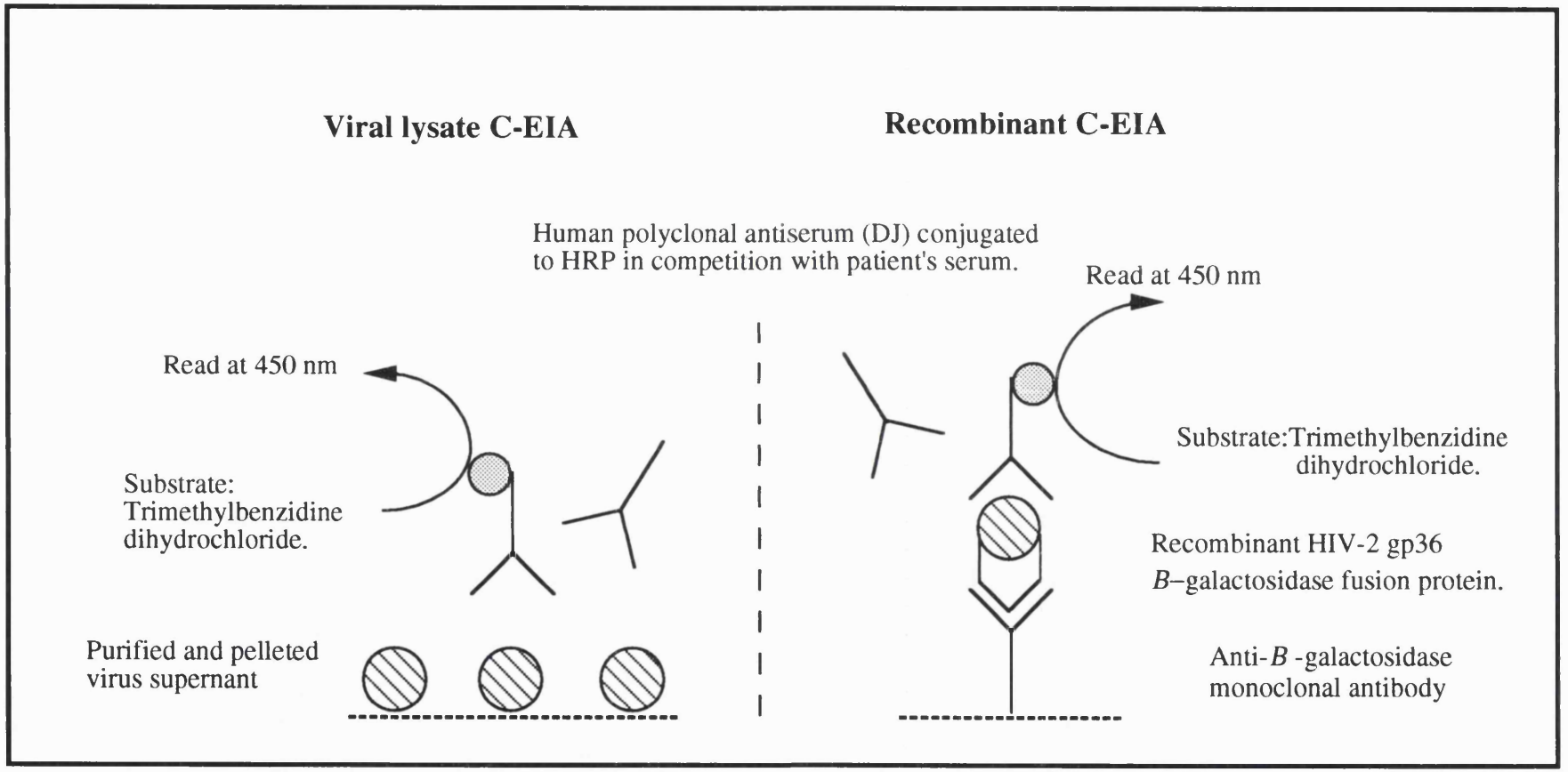


Figure 2.1. Comparison of solid-phase formats of competitive assays for anti-HIV-2 detection.

Assays can be performed in parallel using 25µl of test serum with the VC-EIA and 50µl of test serum with the RC-EIA and 75µl of reconstituted DJ conjugate.

hydrolysis, was performed by incubation at 37°C. The pH was adjusted from acidity (straw-yellow) back to neutrality (pink) at approximately half-hourly intervals using 100mM NaOH until no further colour change occured. Differing volumes of inactivated supernatant fluid, up to three litres, were filtered and concentrated through 0.45μM and 0.1μM filters respectively (Millipore Ltd) and pelleted through a linear 20% sucrose cushion in TEN buffer (Appendix 1) at 52 000g for two hours using an SW-50 swing-out rotor in an L-20 ultracentrifuge (Beckman Ltd). Pelleted virus was resuspended in 500μl TEN buffer and stored at -70°C.

Antigen was coated in 100μl aliqouts directly onto Nunc U-bottomed microwells (Gibco-BRL, Paisley, Scotland) in 10mM Tris-HCl, pH 7.6. For each antigen preparation, titrations from 1/100 to 1/800 were performed to determine empirically the optimum coating dilution. Plates were kept in a moist chamber for 2-3 days at room temperature and washed six times with a three minute soak in Tween-saline wash buffer (Appendix 1) followed by a further six washes. A 150μl aliquot of a 1% aqueous solution of degraded gelatin was added to each well for one hour, after which wells were aspirated and dried at 37°C for 2 hours. Solid-phase was stored in the presence of a dessicant at 4°C until required.

iv. Assay protocol.

The anti-HIV-2 conjugate used throughout these studies was a human polyclonal serum conjugated to horseradish peroxidase (HRPO). The original source of the serum was an HIV-2-infected Gambian patient designated DJ from whom a relatively large volume of serum was obtained. The conjugates, both DJ and other anti-HIV-2 sera, were prepared as previously described (Duncan *et al*, 1983) and were gifts from Dr J. Duncan, Murex Diagnostics Ltd. The optimal dilution of DJ conjugate had been previously determined (Tedder *et al*, 1988) using CBL.20 antigen and was stored long-term in a freeze-dried form. Vials of freeze-dried conjugate were reconstituted with 9mls of conjugate diluent (Appendix 1).

Strong positive and cut-off control sera which gave >90% and 50% inhibition in the assay respectively were included. These were 1/25 and 1/7000 dilutions of DJ serum in

normal human serum (NHS, free from all HTLV, HIV and HBV markers) containing 0.2% Bronidox solution and NHS as a negative control. All reagents stored at 4°C were allowed to equilibrate to room temperature for 20 minutes prior to use. To each of the first eight wells 25µl of serum were added followed by 75µl of conjugate, avoiding contaminating the conjugate with test serum. Plates were incubated in a waterbath for 1 hour at 37°C, aspirated on an automatic plate-washer (Skatron Ltd) for six wash cycles followed by a two minute soak and a further six washes. Trimethylbenzidine dihydrochloride (TMB) substrate was reconstituted in substrate diluent (Appendix 1) and 100µl aliquots reacted to visualise bound conjugate. Colour reaction was stopped after 20 minutes with 50µl 2M H_2SO_4 and read on an automated microplate reader (Multiscan, Flow laboratories) at a wavelength of 450nm.

2.a.2. Recombinant competitive EIA (RC-EIA).

i. Expression of HIV-2 transmembrane glycoprotein.

Immunodominant domains of the transmembrane glycoprotein (gp36) of an HIV-2 strain isolated in Belgium (ANT 53) were cloned and expressed in *E.Coli* as previously described (Parker *et al*, 1989) by Dr. D. Parker and colleagues, Murex Diagnostics using standard protocols (Sambook *et al*, 1989). Briefly, a cDNA clone (DM319), was derived from the HIV-2 region equivalent to the immunodominant area of HIV-1 (Ho *et al*, 1987; Modrow *et al*, 1987) and was subcloned into the expression vector pDEV107. The resulting plasmid (pDM999), when induced in *E.Coli* TG-1 cells, expressed the HIV-2 polypeptide as a β-galactosidase fusion protein. The region expressed (Figure 2.2) includes the highly immunodominant region of HIV-2, in particular the WGCAFR motif (Norrby *et al*, 1987). Overnight cultures of TG-1 cells containing the plasmid pDM999 were diluted 5-fold with fresh media (L-Broth containing 100µM amplicillin) and incubated at 37°C until the culture reached an OD of 1.0 at a wavelength of 600nm. Cultures were induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 50µg/ml final concentration) and the incubation continued for a further 3 hours. Cells were recovered and lysed using lysozyme/DNAase treatment (Parker *et al*, 1989). Crude antigen extracts were disrupted by sonication, aliquoted and stored at -70°C until required.

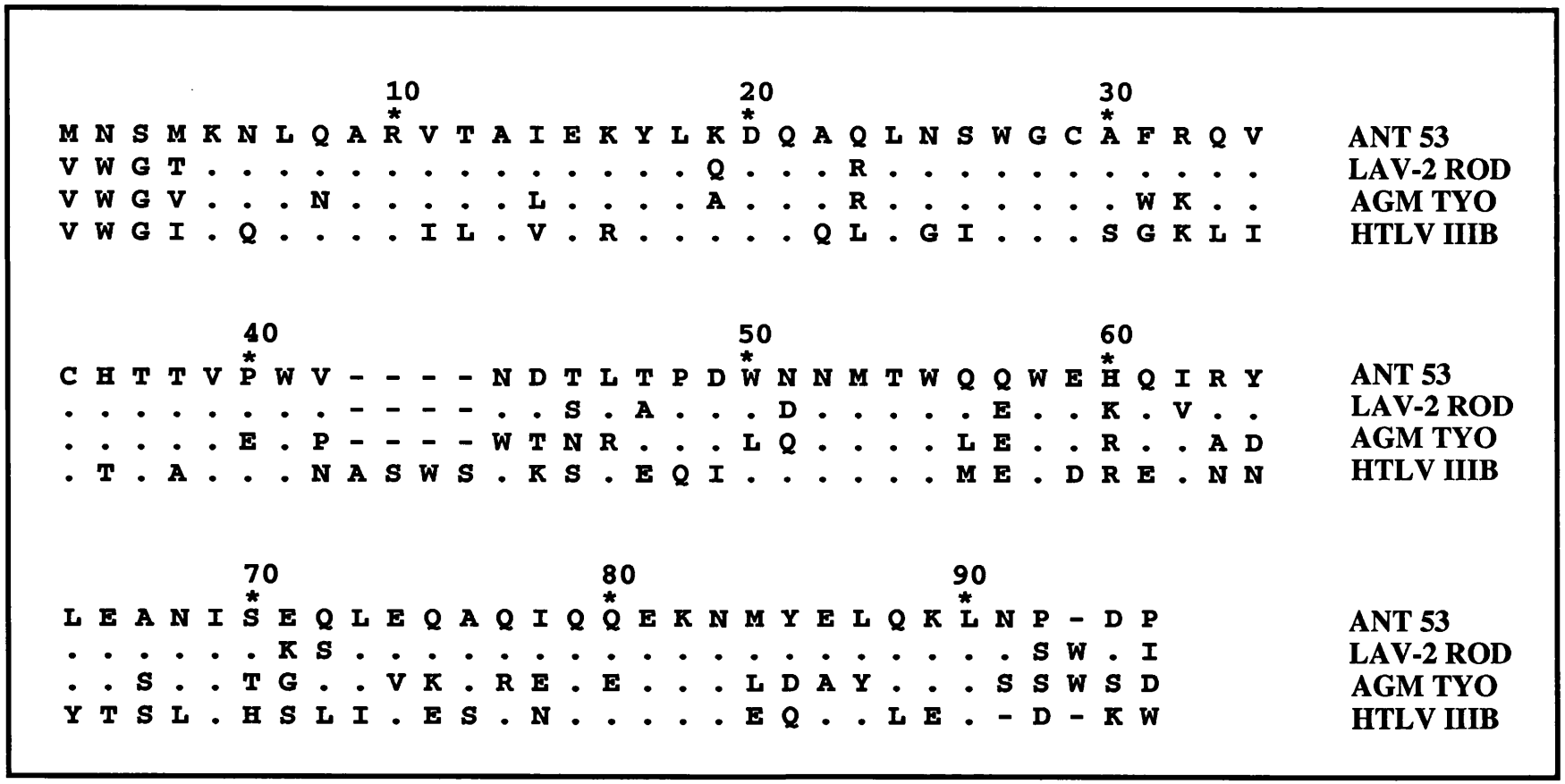


Figure 2.2. Amino acid sequence of the cloned region of the HIV-2 transmembrane glycoprotein. The European HIV-2 isolate (ANT 53) designated 999/164 compared to the equivalent region of the prototype strains of HIV-2 (LAV-2 ROD), HIV-1 (HTLV IIIB) and SIV AGM (TYO). Artificial amino acids introduced for cloning purposes are MNSM- and -PDP at the amino and carboxy ends respectively. Amino acids 16-35 represent the highly immunodominant domain.

ii. Purification of anti-β-galactosidase monoclonal.

The antibody used to capture the β-galactosidase fusion protein was a monospecific antibody raised to β-galactosidase (designated BG-79) and was purified from ascites, a gift from Murex Diagnostics Ltd, by ion exchange chromatography. Ascitic fluid (2-3 mls) was clarified, the supernatant decanted and diluted 1:1 in 10mM phosphate buffer, pH 8. An equal volume of saturated ammonium sulphate solution was added dropwise on a vortex and then centrifuged at 3000-4000g for 20 minutes at 2-8°C. Supernatant was discarded and the pellet resuspended in 10mM phosphate buffer, pH8 to half the original volume of ascites and dialysed (X3) with 10mM phosphate buffer, pH8 (200 volumes).

A DEAE Sepharose (DEAE-Sephacel, Pharmacia Ltd) column with a bed height of 10cm was prepared and equilibrated with 10mM phosphate buffer, pH8 and dialysed antibody eluted onto the column at an approximate flow-rate of 0.5 ml/minute using a peristaltic pump attached to a Uvicord tracer (LKB/Pharmacia Ltd). Elution was continued to baseline followed by further step-wise elution with 20mM phosphate buffer, pH 8. Antibody was eluted off as a single peak, collected and dialysed two times against 100 volumes of 0.85% saline. The column was regenerated with 0.3M phosphate buffer, pH 8 followed by two column volumes of 0.1M NaCl and stored in 10mM phosphate buffer containing 0.1% sodium azide. Typically, 15-20 mls of antibody eluted in the first peak was concentrated to 1-2mls using a diposable concentration unit (Millipore Ltd). Immunoglobulin concentration was determined by spectrophotometry (1mg/ml IgG has an absorbance of 1.3 at 280nm).

iii. Preparation of solid phase.

Aliquots (100µl) of BG-79 capture antibody at a concentration 5µg/ml were coated onto Nunc U-bottomed micowells and incubated at room temperature for 12-16 hours. Wells were washed with Tween-saline buffer and quenched with 10mM Tris-HCl, pH 7.6 containing 0.2% BSA for 1-2 hours at room temperature or at 4°C for several days. Aliquots of crude recombinant antigen, stored at -70°C, were solubilised with 10% SDS (final concentration of 1-2 %) for 30 minutes at 37°C. The extremely insoluble nature of the fusion protein required extensive denaturation with SDS since other treatments

such as urea were ineffective at solubilising this protein. Recombinant antigen was diluted in 10mM Tris-HCl, pH 7.6 containing 1% Nonidet P-40. Optimum concentrations of antigen were determined empirically by making 100µl aliquots of doubling dilutions of 1/1000, 1/2000, 1/4000 and 1/8000 which were captured onto the solid-phase for 16-36 hours at room temperature. Solid phase was washed, blocked and stored as described in Section 2.a.1.iii. Competitive assay formats are compared in Figure 2.1. Assay formats are described in section 3.a. Figure 3.1.These may be broadly classified as:

Type 1:	**Antiglobulin.**	**Type 2:**	**Competitive.**
Type 3:	**Reverse capture.**	**Type 4:**	**Immunometric.**

2.a.3. Alternative serological assays.

A brief description of the assay format and antigen composition of monotypic and combination asays is given below:

i. Monotypic assays.

Wellcozyme HIV recombinant (Murex Diagnostics Ltd, Dartford, UK).

Competitive assay for HIV-1 antibodies employing a recombinant antigen as a *gag-env* fusion protein immobilised to the solid-phase by an anti-*gag* monoclonal antibody (Ferns *et al*, 1987). Anti-HIV-1 peroxidase-conjugated human antibody, visualised by TMB.

Elavia 2 (Diagnostics Pasteur).

Antiglobulin assay for anti-HIV-2; two wells per sample, one coated with purified virus antigen and the other with a mixture of cellular and serum antigens, detected by peroxidase-conjugated polyclonal anti-human IgG and O-phenylenediamine (OPD).

ii. Combination assays.

Abbott recombinant HIV-1/HIV-2 EIA (Abbott Diagnostics Ltd).

Antiglobulin assay with recombinant antigens of the *gag* and *env* of HIV-1 and HIV-2 *env* coated on polystyrene beads. Detection is by a peroxidase-conjugated anti-human IgG and OPD.

Enzygnost anti-HIV-1 and HIV-2 (Behring Ltd).

Antiglobulin assay where three synthetic peptides of the envelope of HIV-1 and one of HIV-2 are coated on the solid phase. Detection is by a peroxidase-conjugated anti-human IgG and TMB.

Elavia Rapid Mixt (Diagnostics Pasteur).

Antiglobulin assay using purified cell-culture antigens of HIV-1 and HIV-2 coated on the solid phase. Detection is by a peroxidase-conjugated anti-human IgG and OPD.

Wellcozyme combined HIV-1 and HIV-2 (Murex Diagnostics Ltd).

Immunometric assay using recombinant *gag* and *env* antigens of HIV-1 and synthetic peptides of HIV-2 *env* coated on the solid phase. Detection is by the same antigens conjugated to alkaline phosphatase. Catalysis of the dephosphorylation of NADPH to NADH acts as a substrate for IQ BIO amplification reaction, also shown in Figure 2.7.

iii. Western blot.

Confirmatory testing for anti-HIV-1 and anti-HIV-2 was performed using either Pasteur or DuPont (Du Pont de Nemours Ltd) Western blot kits in accordance with the manufacturers' instructions. Western blot assays are antiglobulin in format with antigen derived from whole virus preparations.

iv. Interpretation of optical densities.

Optical densities (OD) were expressed as normalised ratios to allow inter-test comparisons of data. For assays where a positive reaction was indicated by an increased OD ie antiglobulin and immunometric assays, normalised EIA ODs were derived by dividing the test OD by the cut-off OD. For assays where a positive reaction was indicated by a decreased OD, ie competitive EIAs, normalised ODs were derived by dividing the cut-off OD by the test OD such that positive sera always had a normalised OD ratio of >1 and negative sera an OD ratio of <1. Positive results were graded as weak (ratio of >1 and of <5) or strong (ratio of >5) to enable results of antiglobulin assays (ELAVIA-2) to be compared with competitive assays (VC-EIA). For comparisons of the results obtained in different competitive assays with various sera

either standardised optical densities (calculated by dividing the test OD value by the cut-off OD value) was used or the percentage inhibition of the conjugate determined (by subtracting the test value from the mean of the negative controls and dividing by the mean values of negative controls minus the mean of the positive controls, expressed as a percentage). Sera with greater than 90% inhibition were considered to be strongly reactive with a cut-off value imposed at 50% inhibition.

2.a.4. Patients and sera.

i. Evaluation of VC-EIA.

The VC-EIA was evaluated both in London and in field studies at the MRC laboratories, The Gambia over a period of 3-4 years on number of different panels of sera:

Panel 1. A random sample of 491 adults were selected from a rural area of The Gambia which had previously been documented (Wilkins *et al*, 1991a). Sera were screened with the competitive assay and the ELAVIA-2 assay to allow a comparison of the prevalence of positive reactions.

Panel 2. Two hundred and fifty women bled during a study of prostitutes working in The Gambia were screened twice with the competitive assay to assess the reproducibility of results. The second test was carried out without reference to the first.

Panel 3. The relationship between the results of the two EIA's and confirmatory Western blot was also investigated. Four hundred and thirty sera from a variety of sources were used, including surveys of the general population and high-risk groups including prostitutes and STD patients as well as from patients suspected of having HIV-related disease.

ii. Determination of sensitivity by end-point dilution.

For sensitivity estimates, a master dilution series was made for each serum used in the evaluation by titrating sera reactive for anti-HIV-2 across their end-points in NHS. Each dilution, treated as single serum specimen, was then tested in both the VC-EIA and RC-EIA and interpolated end-points derived which were used as a measure of the limiting

antibody concentration detectable in each assay. In addition, a series of four seroconversion sera were obtained from a single Gambian individual (provided by Dr. A. Hughes, Royal Victoria Hospital, Banjul, The Gambia). Titration to end-point of these samples represents an indication of the ability of assays to detect low avidity, acute-phase antibody.

iii. HIV-1 and SIV sera.

HIV-1 serum samples were obtained from colleagues within the diagnostic service in the Department of Virology, UCLMS and The Gambia for studies of cross-reactions with HIV-2 antigens. Serum samples were also obtained from ten African green monkeys some of which were obtained from feral populations in the Central African Republic (kindly provided by Dr. F. Barre-Sinoussi, Institute Pasteur, Bangui, Central African Republic). These were reacted undiluted in each of the competitive assays (anti-HIV-1 and anti-HIV-2) and the relative antibody titres of reactive sera determined by end-point dilution and the percentage inhibition of the conjugate determined for each. A serum sample obtained from a macaque infected with the SIV_{mac} (32H strain), obtained through the MRC AIDS Directed programme and a serum sample from an SIV-infected sooty mangabey (SIV_{sm}) were also tested by HIV-2 competitive EIA.

2.b. Application of PCR-based techniques.

Nested PCR assays were developed for the amplification of HIV-2 proviral DNA and viral RNA from clinical material using samples provided by colleagues at the MRC laboratories, Fajara and the Royal Victoria Hospital, Banjul. The techniques described are adaptations of the original PCR methods for the *in vitro* amplification of specific DNA sequences (Mullis and Faloona, 1987; Saiki *et al*, 1988) and have been optimised for the efficient detection of HIV-2 sequences.

2.b.1. Design, synthesis and preparation of oligonucleotide sequences.

Eight HIV-2 isolates where the entire genomes had been cloned and fully sequenced as described in Section 1.b.2.ii. and recorded on the Los Alamos Database were analysed using Microgenie software (Beckman Instruments Ltd). Targets for PCR amplification were part of the U3/R region of the LTR, a conserved part of the integrase gene within *pol*, the *vpx* gene and a region extending into the *nef* gene from the 3' LTR. *Nef* primers (sense) were used in conjunction with the antisense LTR primers and application of this primer set is described in Chapter 6. The *gag* gene of HIV-2 has also been studied and is described in Section 2.c. and Chapter 4. Oligonucleotide primer identity and source for primers used in detection and quantification studies are described for HIV-2 in Table 2.1 and in Table 2.2. for HIV-1. A diagramatic representation of the regions used solely for diagnostic purposes is shown in Figure 2.3.

Experiments were first performed with oligonucleotide primers synthesised on a synthetic oligonucleotide synthesiser (Applied Biosystems Ltd). Following a 0.2μmole synthesis, primers were extracted from a CpG column using fresh 30% (v/v) ammonia (BDH Ltd) at 4°C. Oligonucleotides were washed through the column three times and collected in a glass vial with a teflon-coated cap (Wheaton, supplied by Jencons Ltd.). Deprotection and cleavage of the protective groups was achieved by incubation at 55°C overnight and cooling to 4°C.

Primers were prepared for use by precipitation with absolute alcohol, 1/10th 3M sodium acetate, pH4 at -70°C overnight. Where a biotin moiety was required, primers were synthesised with an amino group (amino-link) to allow 5'-OH derivatisation and

Region amplified	Orientation (sense/antisense)	Sequence (5' to 3')	Length (-mer)	Location (nt)[a]	Reference/ Source	Predicted size (bp)
LTR	+ outer	TAACCAAGGGAGGGACATGGG	21	9411-9431	Myers *et al*, 1990[c]	181
"	- outer	TGGTGAGAGTCTAGCAGGG	19	9574-9592	"	
"	+ inner	AGGAGCTGGTGGGGAACGCCCT	21	9432-9453	"	141
"	- inner	AACACCCAGGCTCTACCTGCT	21	9553-9573	"	
nef[b]	+ outer	TGGCAATAGATATGTCACATTTTATAAA	28	8900-8927	Gao *et al*, 1992	692
"	+ inner	CCAGAAGAGTTTGGGCACAAGTCAGG	26	9244-9269	Myers *et al*,1992	329
UNIPOL 1	+ inner	AGTGGATTCATAGAAGCAGAAGT	23	4298-4320	Miura et al, 1990	336
" 2	- outer/inner	CCCCTATTCCTCCCCTTCTTTTAAAA	26	4609-4634	"	
" 3	+ outer	TGCACACATTTAGAAGGAAA	20	4250-4269	Myers et al, 1990	384
pol	+ outer	GCAGTICATGTIGCIAGTGG	20	4283-4302	Myers *et al*,1990	358
(integrase)	- outer	GTCATATCCCCTATTCCTCCCC	22	4620-4641	"	
"	+ inner	AGTGGATTIATAGAIGCAGA	20	4298-4317	"	311
"	- inner	ATTCATGCAATGAATTGCCAT	21	4598-4609	"	
vpx	+ outer	AGGTACCGTCACTTCAATTTCTGG	24	5299-5322	Myers *et al*,1989	453
"	- outer	TGAGGGAGCCAGGGGATGAGTGGAT	25	5728-5752	"	
"	+ inner	CCTTAGTGGTAGTGCAACAAAA	22	5323-5344	"	379
"	- inner	GTCTAATGGCTGAAGCACCAACAGAG	26	5677-5702	"	

Table 2.1. Oligonucleotide sequences for nested amplification of the HIV-2 genome. [a]Sequences numbered according to HIV-2 ROD, GenBank accession number M15390. For the *pol* (integrase), but not UNIPOL primers an I denotes a neutral inosine residue at positions where there is recognised variability of HIV-2 strains. UNIPOL 2 (antisense) was also used in first round with UNIPOL 3 and second round with UNIPOL 1. [b]*nef* primers (sense) were used in conjunction with LTR antisense sequences to yield the predicted product sizes with HIV-2 strains. [c]Myers et al, indicates oligonucleotides designed by the author from the Los Alamos database of the year indicated.

Region amplified	Orientation (sense/antisense)	Sequence (5' to 3')	Length (-mer)	Location (nt)[a]	Reference/ Source	Predicted size (bp)
pol (reverse transcriptase)	+ outer	GCAGGGGCAAGGCCAATGGACAT	23	3541-3563	Kaye *et al*, 1991	252
	- outer	CTCCCACTCAGGAATCCAGGTGGC	24	3770-3793	"	
	+ inner	CAGGAAAATATGCAAGAATGAGG	23	3600-3622	"	143
	- inner	CCCATGTTTCCTTTTGTATGGGT	23	3722-3743	"	
vpu	+ outer	ATCTCCTATGGCAGGAAGAAG	21	5962-5982	Myers *et al*, 1989	384
	- outer	TTGTGGGTCACAGTCTATTATGGG	24	6323-6346	"	
	+ inner	CAAAGCAGTAAGTAGTACATGTA	23	6038-6060	"	286
	- inner	GATCTGTAGTGCTACAGAAAAATT	24	6201-6324	"	

Table 2.2. Oligonucleotide sequences for nested amplification of the HIV-1 genome. The 1990 edition of the Los Alamos database was used to design the *vpu* primers. [a] Sequences numbered according to HIV-1 HXB2, Genbank accession number K03455.

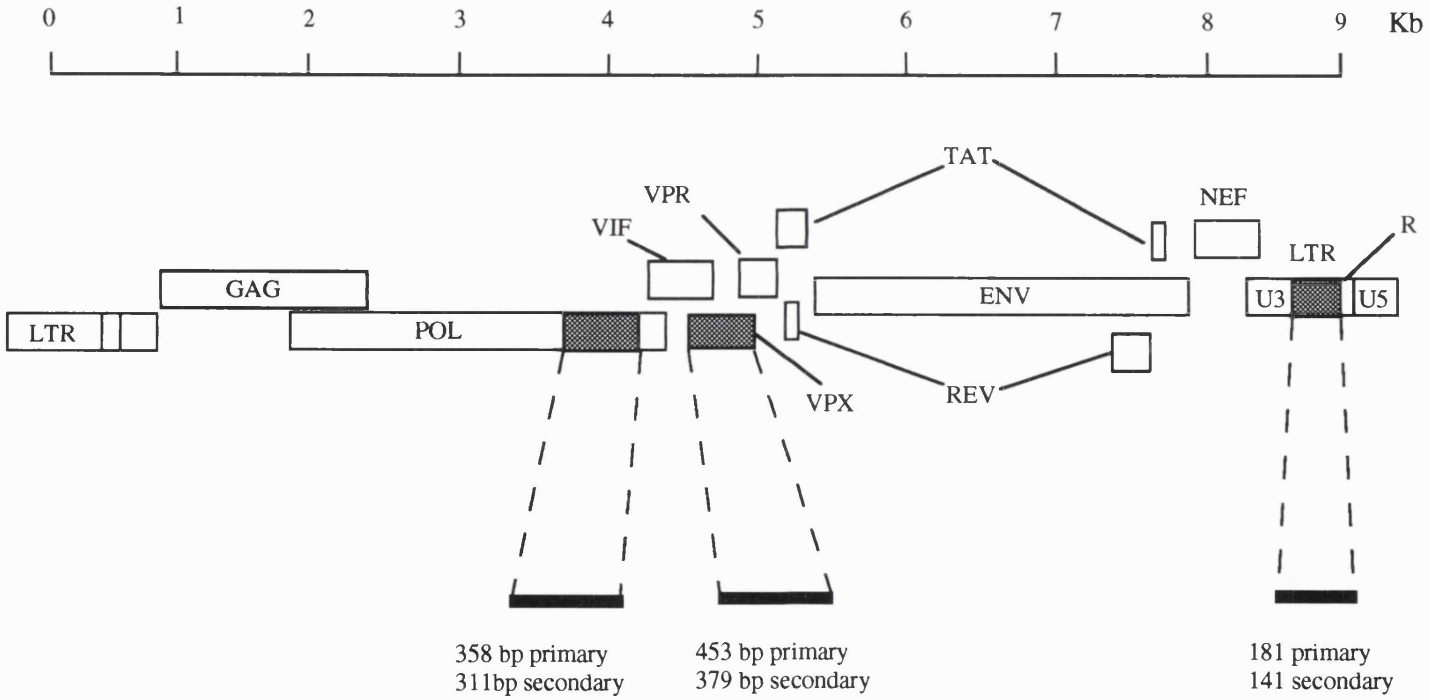


Figure 2.3. Regions of the HIV-2 genome targetted for diagnostic nested PCR amplification. The product sizes shown for part of the integrase gene in *pol* are for the modified fully nested primer set.

conjugation of free biotin moieties in the presence of N-hydroxysuccinimide ester and dimethyl formamide (Sigma Ltd). Unbound biotin was removed by dialysis. Approximate yield and primer concentration was determined by spectrophotometry whereby an OD of 1 is equivalent to 33µg/ml. Primers were also obtained from commercial sources (Oswel DNA unit, Edinburgh University; Pharmacia Ltd). All oligonucleotides were less that 50 bases in length and the theoretical melting temperature (Tm) calculated according to:

$$Tm = 4\ (G+C) + 2\ (A+T) \text{ minus } 5°C.$$

2.b.2. Extraction and quantification of cellular DNA.

i. HIV-infected cell lines.

A panel of DNA extracts were prepared from a range of virus isolates previously established in continuous cell lines. These were four Gambian HIV-2 strains (CBL.20-23) isolated at the Institute for Cancer Research, Chester Beatty Laboratories, London (Schulz *et al*, 1990), LAV-2_{ROD} (Clavel *et al*, 1986a), SIVmac32H (obtained from the MRC ADP reagent repository, NIBSC, Potters Bar) and 11 HIV-1 isolates of different geographical origin, SF33, Z84, ARV4, HTLV-IIIB, CBL-1, RF, K12, ARV2, U455a, GB8, and MN2 obtained from various sources and held in the Department of Virology.

Each isolate was cultured as described in Section 2.a.1.i. in 25cm^2 culture flasks, cells spun and washed in PBS and the pellet resuspended in a lysis buffer containing SDS and proteinase-K (Appendix 1). Cells were simultaneously lysed and inactivated by incubation at 65°C for 2 hours followed by two extractions with phenol:chloroform. Equilibrated phenol was prepared by mixing water-saturated phenol (Rathburn Ltd,) with 10mM Tris-HCl, pH 7.6 and following at least one change with Tris buffer, the pH checked accordingly. Chloroform (AnalR grade, BDH Ltd) and isoamylalcohol (24:1) were added to the equilibrated phenol at a 50:50 ratio. A further chloroform extraction was performed to remove trace amounts of phenol and DNA precipitated with absolute alcohol and 1/10th 3M sodium acetate, pH4 at -70°C for 1 hour or at -20°C overnight. DNA was pelleted in a bench microfuge at 13000rpm for 30 minutes, washed with 70% ethanol and resuspended in half concentration TE buffer (Appendix 1). The

concentration of DNA extracted for each isolate was determined by spectrophotometry (Philips Ltd), whereby an optical density of 1 was equivalent to 50μg/ml at 260nm.

ii. Patients and samples.

An initial set of 23 PBMC samples originating from HIV-2 seropositive and seronegative prostitutes from the Faraffeni region of The Gambia were used in early studies. In later studies, a further 101 samples were obtained from 86 HIV-2 seropositive patients (5 children and 81 adults) and 30 samples from seronegative patients recruited from the STD clinic or from the general outpatients ward of the MRC hospital, Fajara. After a thorough clinical and laboratory examination, performed by clinical colleagues at MRC Fajara, the adult patients were classified into one of three clinical categories according to the 1993 CDC revised classification system (CDC, 1993). Category A included patients who were asymptomatic or had persistent generalised lymphadenopathy. There were no patients with acute primary infection. Category B included patients who were symptomatic but did not have AIDS and Category C included patients with AIDS as well as those with pulmonary TB. Informed consent was obtained from each patient and enrolment protocols were approved by The Gambian Government/Medical Research Council Ethical committee and The Gambia National AIDS committee.

The percentage of CD4+ cells was estimated from whole heparinized blood by use of a fluorescent antibody cell-sorter (FACS) machine after staining with the appropriate monoclonal antibodies (Becton-Dickenson, U.K), performed by collaborators in The Gambia. The total white cell count and the percentage of lymphocytes were estimated by microscopy, the absolute CD4 count derived and the percentage of CD4+ cells obtained by FACS analysis.

iii. Rapid method for preparation of DNA.

The method described by Higuichi, (1989) was used. PBMCs had been isolated using Lymphoprep (Mycomed Ltd) in The Gambia, transported to London on dry ice and stored at -70°C in freezing medium (Appendix 1). Each aliquot of frozen cells was thawed rapidly in a 37°C water bath and transferred to a conical bottomed 1.5ml

microfuge tube (Sarstedt Ltd) in a class 1 safety cabinet. Cells were pelleted at 5000rpm for 2 minutes and the freezing medium removed using a fine-tipped pastette, washed with 1ml sterile PBS, respun and excess PBS removed. To each sample, 200µl of DNA extraction buffer (Appendix 1) was added and after a brief vortex, incubated at 60°C for 2 hours. Even digestion of cellular protein was obtained by vortexing the tubes briefly after 1 hour when potentially infectious virus had been rendered inactive. After 2 hours, the samples were incubated at 95°C for 10 minutes to inactivate excess proteinase K, aliquoted and stored at -20°C.

iv. Measurement of DNA concentration by fluorometry.

The concentration of DNA in a sample extracted by the rapid lysis method cannot be measured by standard spectrophotometry due to the high concentration of protein in the sample. DNA concentrations were therefore measured by fluorometry. A TK100 series DNA mini-fluorometer (Hoefer Instruments Ltd) was standardised using calf thymus DNA (Boehringer Ltd). A stock of 1mg/ml was diluted in 1X TEN buffer to 250 and 100µg/ml to make DNA standards. Hoechst 33258 dye was made as a 1mg/ml stock solution and stored in the dark at 4°C. A 10X TEN stock solution was diluted 1 in 10 in distilled water and 10µl of stock dye added. Two microlitres of standard or sample were diluted in 2ml of 1X TEN buffer containing dye and all measurements made using the same cuvette. Once calibrated for each series of samples to be measured, no conversion of DNA content is required since the reading from the fluorometer is a direct measurement in µg/ml. Each sample was adjusted to 60µg/ml for either qualitative or quantitative PCR.

2.b.3. Nested amplification of HIV-2 proviral DNA.

i. Pre and post-PCR manipulations.

All PCR reactions were nested in format and performed according to recommended guidelines established for PCR within designated work areas (Kwok and Higuchi, 1989). This consisted of a pre-PCR area free of amplified products and a contained post-PCR area where all amplification reactions and subsequent handling of PCR products were performed. For each set of primers, amplifications were performed in 0.5ml disposable tubes (Sarstedt Ltd) containing, typically, 50µl volumes of a reaction

mixture containing 50mM KCl, 10mM Tris-HCl pH 9, 0.1% Triton X-100, 1.5mM $MgCl_2$, 200μM of each dNTP (dATP, dGTP, dCTP, dTTP), 10 pmoles of each external primer and 1 unit of *Taq* DNA polymerase (Perkin-Elmer/Cetus Corporation; Promega Ltd). Cycling parameters were 94°C denaturation for 4 minutes for 1 cycle, 94°C denaturation for 1 minute, 50°C anneal for 1 minute and 72°C extension for 1 minute for 35 cycles, and a final extension for 7 minutes at 72°C. A 0.5-10μl positive displacement pipette (Eppendorf, BDH Ltd) was used to transfer 1μl aliquots from first to second round reaction mixes prepared with fresh reagents and the internal primers. Cycling was continued for 25 cycles following 94°C denaturation for 1 minute, 50°C anneal for 1 minute and 72°C extension for 1 minute. Amplification reactions were performed on a DNA thermal cycler (Hybaid Ltd).

ii. PCR controls.

The reactivity of the modified *pol*, LTR and *vpx* primers (HIV-2), the *vpu* primers (HIV-1) and the UNIPOL primers (HIV-1, HIV-2) were determined using a combination of the different control samples. Preparations of Hirt DNA (CBL-20-23) and DNA extracted from culture-derived isolates of CBL.20-23, LAV-2_{ROD}, SIVmac251 (32H isolate) and HIV-1 isolates (section 2.b.2.i) were also used. The modified plasmid (pROD$_{10}$ Δ *gag-pol*) containing both 3' and 5' LTR sequences was also used to assess the efficiency of the LTR primers only. Both the Hirt DNA preparations and the plasmid were provided by Dr. T. Schulz, ICR, Chester Beatty Laboratories, London. For studies of HIV-2 subtype B viruses, two strains were used. Plasmid DNA of the prototypic subtype B virus HIV-2_{D205} was obtained from Dr.U. Dietrich, Georg Speyer Haus, Germany. HIV-2_{7312A}, obtained from Dr. B.Hahn, University of Alabama, USA has a subtype B LTR cloned into pBluescript SK+/- (Stratagene Ltd).

iii. Analysis of PCR-amplified products.

PCR-amplified DNA fragments were routinely analysed by agarose gel electrophoresis. Depending on the size of the DNA fragment, various concentrations of gel were used. Typically for 100-700bp fragments a 1.5% agarose gel in 1X TAE buffer (Appendix 1) containing 0.5μg/ml ethidium bromide was used and electrophoresed for 45-60 minutes

at 80-120 volts in 1X TAE buffer and viewed under an ultra-violet transilluminator (Genetic Research Instruments Ltd). Analysis of amplified fragments where small differences in the expected fragment size were observed (20-40bp deletions of the HIV-2/SIV 3' LTR amplified as a 350 bp fragment) gels with greater resolving powers were used. MetaPhor™ agarose (Flowgen Ltd) was used to make a 2-3% gel, prepared by dissolving in 1X TAE buffer and heating in a microwave oven until dissolved and then cooling to 65°C before pouring. Gels were cooled at 4°C for 30 minutes prior to use.

2.b.4. Quantification of amplified DNA.

i. Radiometric quantitative incorporation assay.

A solid-phase quantitative radiometric incorporation assay for quantification of HIV-2-amplified products was developed in a microwell format. The principle of the assay is described in Figure 2.4. Modifications were made to the secondary amplification reaction following a standard primary PCR with LTR primers for 35 cycles. For each single reaction, three unlabelled nucleotides (dATP, dTTP, dCTP), each at a concentration of 5μM and 5μCi of DNA sequencing grade [^{35}S] labelled αdATP (specific activity 1400Ci/mmol, DuPont/NEN Ltd) were incorporated into the secondary amplification mixture. Secondary reactions were performed in 50μl volumes using one unit of recombinant *Pfu* DNA polymerase (Stratagene Ltd), substituted in place of *Taq*, in 1X *Pfu* reaction buffer (20mM Tris-HCl, pH 8.75, 10mM KCl, 10mM$(NH_4)_2SO_4$, 2mM $MgCl_2$, 0.1% Triton X-100, BSA [100 μg/mL]) and 10pmoles of internal primers. The antisense primer was labelled with a biotin moiety and incorporation was measured following ten rounds of thermal cycling by capturing 30μl of the reaction mix, diluted with an equal volume of PBS containing 0.05% Tween-20, and incubated at room temperature for 45 minutes on streptavidin-coated U-bottom microwells. Microwells had been previously prepared by coating 5μg/ml of streptavidin (Sigma Ltd) in 10mM Tris-HCl, pH 7.6 overnight in microwells and quenched in 10mM Tris-HCl, pH 7.6 containing 0.2% BSA. Plates were stored at 4°C until required for up to one month.

After washing with a vacuum-operated hand-washer, the complementary non-biotinylated strand of DNA was removed with 60μl of 0.15 M sodium hydroxide by

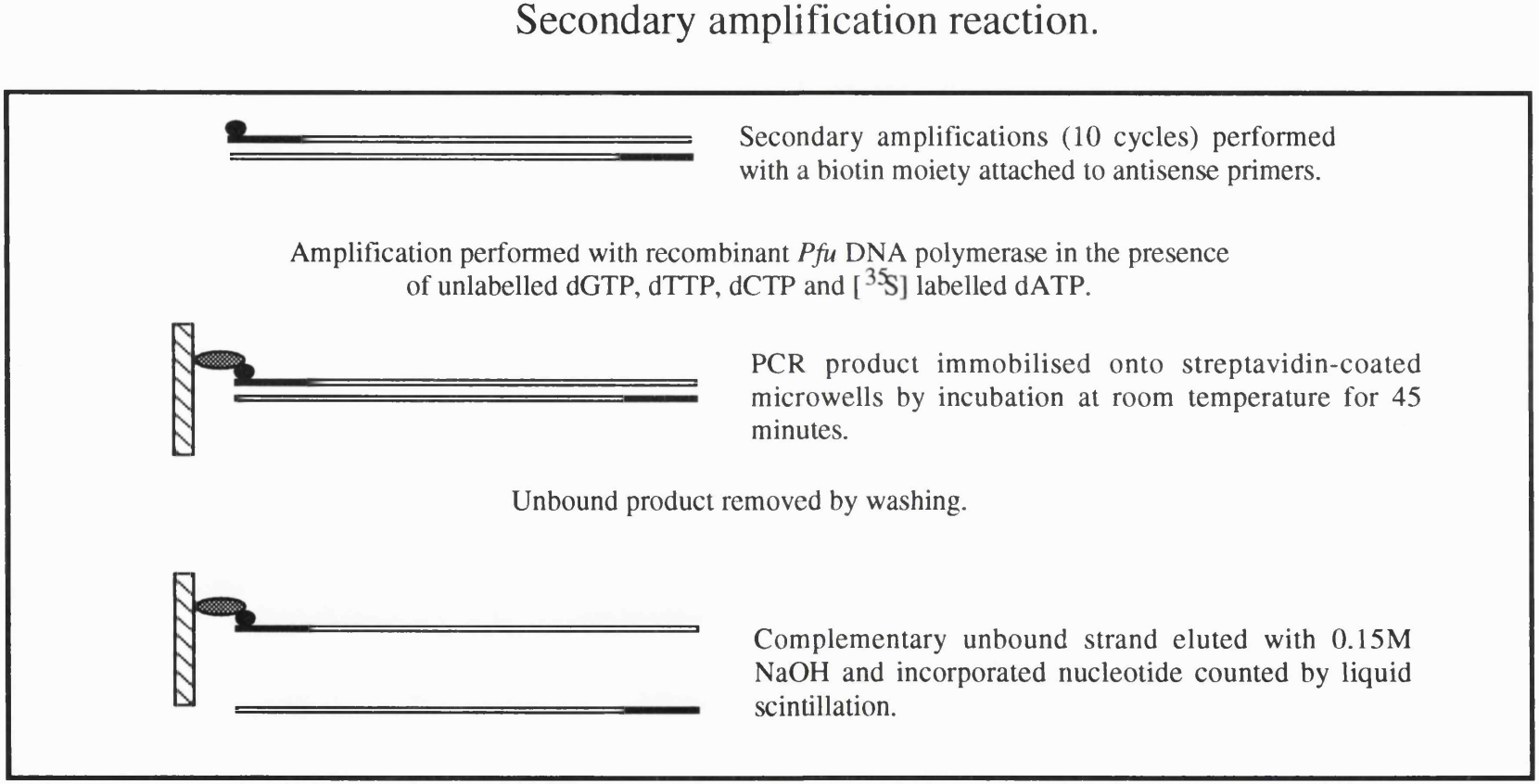


Figure 2.4. Quantitative solid-phase radiometric PCR incorporation assay. Modifications were performed to secondary amplification reactions. A combination of LTR primer sequences, *Pfu* DNA polymerase and radiolabelled nucleotides were used to provide quantitative measurements in a microwell format following ten rounds of secondary amplification.

incubation at room temperature for 10 minutes. Radioactive liquid and solid waste was disposed of according to local guidelines relating to radioactive waste disposal. Fifty microlitres was transferred to 96 well plates (Canberra-Packard Ltd) containing 100µl of 'microscint' liquid scintillant and counted on an automated microtitre β-scintillation counter ('Top count', Canberra Packard Ltd) linked to a microcomputer. For analyses performed in The Gambia, a filter-mat and liquid scintillation system was employed. Aliquots (25µl) of PCR product were spotted onto glass-fibre filter mats (Pharmacia Ltd) and air-dried. These were sealed in a plastic bag containing 10mls of 'Readysafe' scintillation cocktail (Beckman Ltd) and read on a 1205 Betaplate reader (LKB/Pharmacia Ltd) linked to an Olivetti computer and printer.

ii. Quantification by limiting end-point dilution.

HIV-2 proviral DNA copy number was assessed by a limiting dilution method. A master PCR mix was prepared and distributed into 0.5ml PCR tubes in sets of four, to make a dilution series of 1, 1/5, 1/25, 1/125, 1/625, 1/3125 and so forth. Into the first tube containing 95µl of master mix, 5µl of sample DNA was added and vortexed for 15 seconds. Aliquots (20µl) were dispensed into the three remaining tubes, the final 20µl was then transferred into 80µl of PCR mix and 20µl aliquots transferred into the next set of tubes and continued to the final set; 20µl was diluted in 60µl of PCR mix and distributed into the remaining tubes. Nested PCR using the LTR primer set was performed and analysed by gel electrophoresis. Wells were scored positive or negative and the number of DNA molecules calculated according to Poisson probability theory.

The zero term of the Poisson equation was used where conversion of the Poisson formula to its logarithmic form is expressed as:

$$\mu = -\ln Fo$$

where the natural logarithm (ln) of the fraction of negative values (Fo) is proportional to the mean of the number of positive reactions (µ) for each group. The number of molecules per microlitre of DNA was calculated for each control and test samples to provide measurements of copy number of HIV-2 molecules.

2.b.5. Detection of viral RNA in plasma.

i. Isolation of RNA by RNAzol.

This method is essentially that described by Chomczynski and Sacchi, (1987). RNAzol solution was made from stock buffers in the ratio of 1 volume of stock solution A (Appendix 1), 0.1 volumes of 2M sodium acetate pH4, and 1 volume of water saturated phenol (Rathburn Ltd). RNAzol solution (800µl) was added to 1.5ml screw capped tubes (Sarstedt Ltd) and 200µl of serum or plasma added followed by 100µl of chloroform. This mixture was vortexed for 15 seconds and placed on ice for 15 minutes. Organic and aqueous phases were separated by centrifugation for 5 minutes at 4°C (15 000g) in a sealed fixed rotor head (IEC Centra 4-B centrifuge) and 600µl of the upper phase removed. An equal volume of propan-2-ol and 30µg glycogen was added and RNA precipitated at -70°C and isolated by centrifugation (15 000g, 20 minutes). Following one wash with 70% ethanol, the RNA/glycogen pellet was dried by evaporation and dissolved in 10µl of sterile distilled water. 2µl of RNA was used for each cDNA synthesis reaction and the remainder stored at -70°C.

ii. Generation of cDNA.

Complementary DNA (cDNA) was synthesized in all experiments by direct rather than random priming of the reverse transcription reaction using 150-200ng of antisense primer (outer 3' LTR nested PCR primer) per reaction. The cDNA reaction mix consisted of 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$, 0.1M DTT, 10mM of each dNTP, 1 unit of Moloney murine leukaemia virus reverse transcriptase (Pharmacia Ltd) and 30 units of RNA guard (Pharmacia Ltd). First strand cDNA syntheses were performed at 37°C for 60-90 minutes in 20µl volumes and 5µl used directly for PCR and the remainder stored at -20°C.

2.c. HIV-2 *gag* p26 studies.

A series of investigations relating to the p26 gene and gene product (p26 antigen) of the CBL series of HIV-2 strains were undertaken. The results of these studies are described in Chapter 4. The expression studies were performed in collaboration with Dr Helen Mills, formerly at the Institute of Virology, Oxford.

2.c.1.Expression of HIV-2 *gag* p26 in pGEX-3X.

i. PCR amplification of HIV-2 p26 for cloning purposes.

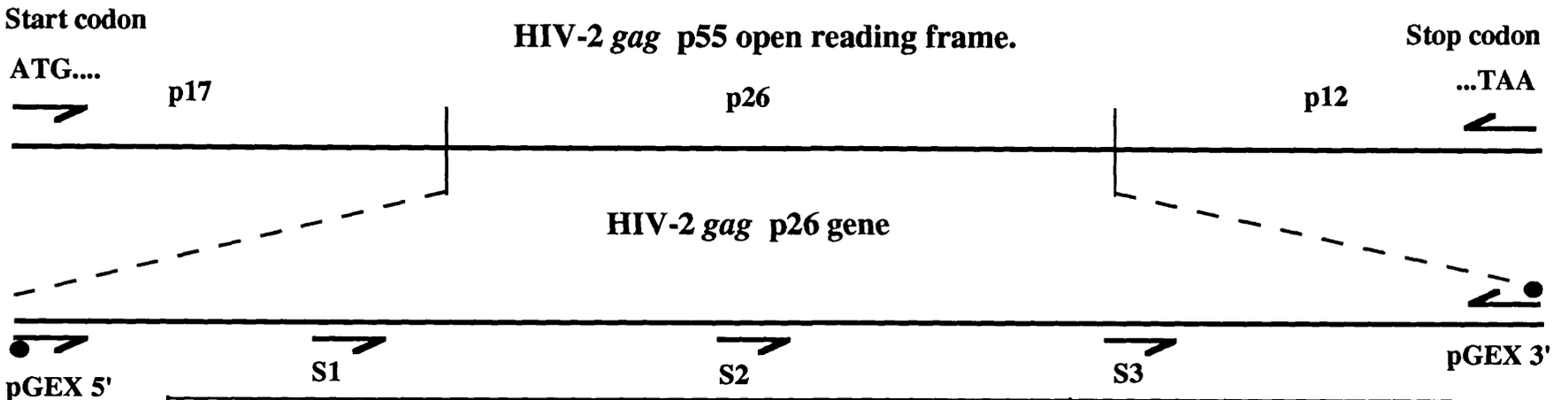
The coding region of the major core protein of HIV-2_{CBL-20} was amplified by nested PCR as shown in Figure 2.5. by primary amplification of the entire p55 gene, followed by secondary amplification of p26. Sequences corresponding to the polyprotein cleavage sites in HIV-2 and SIV strains were used to locate PCR primers. Amplified p26 was subsequently cloned into a polylinker site of the pGEX-3X expression vector (Smith and Johnson, 1988) using modified sequences as described in Figure 2.6. which also shows the pGEX-3X vector sequences and the sites used for cloning purposes. Specific restriction sites for cloning into pGEX-3X were introduced into the p26 primers and a GCG sequence was added to the 5' sense primer adjacent to the *Bam*H1 site and to the 3' antisense primer adjacent to the *Eco*R1 site. The sense primer starts at the TCT (ser) codon since the pGEX-3X sequence will encode the proline residue. Following nested amplification of CBL.20-23 p26, amplified fragments were visualised by agarose gel electrophoresis and staining with ethidium bromide and bands excised from the gel. DNA was isolated by electroelution (IBI Ltd) and the DNA precipitated with absolute alcohol and resuspended in TE buffer.

ii. Cloning of p26 insert into the pGEX-3X vector.

Manipulations were performed using standard protocols (Sambrook *et al,* 1989).

Restriction digestion.

Approximately 1µg of p26 insert was digested with 10 units of *Sma*1 and *Eco*R1 restriction endonucleases in restriction buffer at 37°C for 4 hours. Due to an internal *Bam*H1 restriction site within p26, only *Eco*R1 was used to derive a cohesive end at the



Amplification and sequencing oligonucleotide sequences (5' to 3')	Location (HIV-2ROD)
p55 (outer,sense) ATGGGCGCGAGAAACTCCGTC	546 - 566
p55 (outer, antisense) CTGGTCTTTTCCAAAGAGAGA	2091 - 2111
pGEX p26 S0 (inner, sense) AGTAGACCAACAGCACCATCT	909 - 929
pGEX p26 (inner, antisense) GGCTGCTGCGAATGGGATAGG	1674 - 1694
S1 CAA ATGCTTAATTGTGTGGGCGA	1106 - 1128
S2 AACATCTATAGAAGATGGAT	1329 - 1348
S3 AAGCTTGAGGGCAGAACAAACA	1357 - 1378

Figure 2.5. Amplification of HIV-2 *gag* p55 and nested amplification and sequencing of HIV-2 *gag* p26. The 5' sense and 3' antisense primers in p26 were used as the HIV-2-specific sequences for cloning and expression in pGEX-3X. These sequences were also used in conjunction with a biotin moiety for sequencing of the sense and antisense strands by solid-phase techniques.

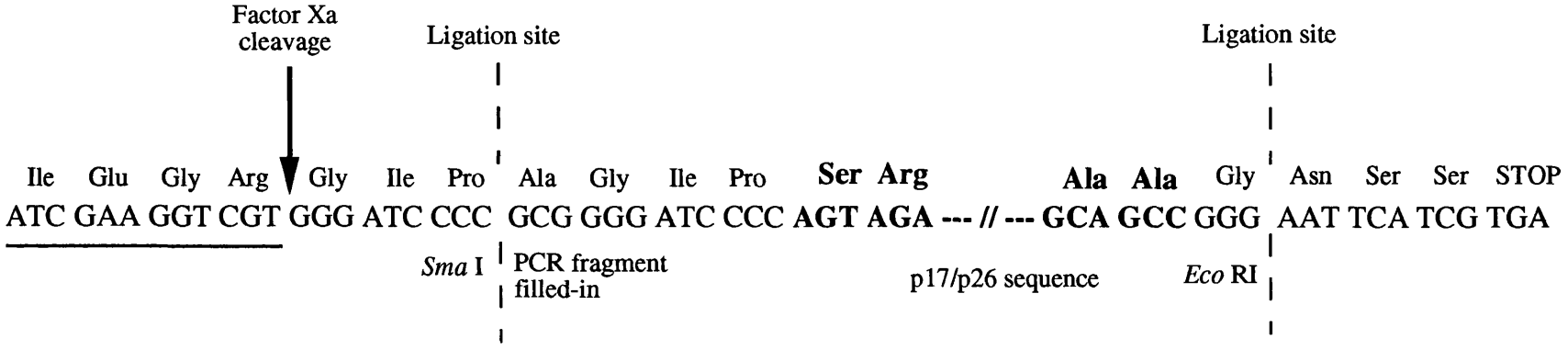


Figure 2.6. Strategy used to clone PCR-amplified HIV-2 p26 into the multiple cloning site of the pGEX-3X expression vector. The fragment encoding p26 was amplified with primers synthesized with internal *Bam* HI and *Eco* RI restriction sites at the 5' and 3' ends respectively. Due to an internal *Bam* HI restriction site within the p26 gene, a 'blunt end' was generated and cloned into pGEX-3X vector using a combination of *Sma*I and *Eco*RI restriction enzymes.

3' end of p26 and the *Bam*H1 site filled to produce a blunt end and cut with *Sma*1. pGEX-3X plasmid DNA was also treated by *Sma*1 and *Eco*R1 digestion.

Dephosphorylation of linearised pGEX vector.

Approximately 0.1 unit of calf intestinal phosphatase (CIP, Boehringer-Manheim Ltd) was added to the digested pGEX DNA and incubated for 30 minutes at 37°C. Unreacted enzyme was inactivated at 85°C for 15 minutes, and DNA isolated by phenol-chloroform extraction and ethanol precipitation and resuspended in TE buffer. pGEX DNA was either used in the ligation reaction or stored at -20°C.

Ligation reaction and transformation of competent cells.

Linearised pGEX DNA and the insert DNA were ligated at a molar ratio of approximately 1:5 in a ligation buffer mix containing 50mM Tris-HCl (pH 7.4), 10mM $MgCl_2$, 10mM DTT, 1mM spermidine, 1mM ATP and 5 units of T4 DNA ligase. Ligation reactions were performed at 10°C overnight and terminated at 65°C for 10 minutes. Five microlitres of ligation mix and 10µl of TE buffer were mixed with JM101 competent cells and placed on ice for 20 minutes. These were then held at 37°C for 5 minutes, an equal volume of TY medium (Appendix 1) added and incubated at 37°C for a further 20 minutes and plated onto agar containing 100µM ampicillin and incubated overnight, upside down, at 37°C. Plates were examined for the presence of colonies which were picked with a sterile tooth pick. The presence of the p26 insert was determined by performing a PCR amplification for 40 cycles with the primers used to initially amplify the fragment. Positive colonies were selected and used in further experiments.

iii. Analysis of pGEX recombinants by SDS-PAGE and Western blot.

At various stages, samples were collected, sonicated on ice and crude preparations electrophoresed on a 12% SDS-polyacrylamide gel using the Protean II mini-gel apparatus (Biorad Ltd). Duplicate gels were run in parallel. The first was stained with Coomassie Brilliant Blue 250 in 50% methanol:10% acetic acid to visualise protein bands and destained in 30% methanol:10% acetic acid. The second was electro-transferred (Western transfer) to nitrocellulose filters and blocked with PBST (PBS

containing 0.05% Tween-20 and 5% Marvel) for 1 hour at room temperature in sealed plastic bags. Blocking buffer (PBST) containing five anti-HIV-2 positive sera diluted 1/100 was added and incubated overnight at 4°C.

Sera were removed and the filter washed in PBS for five minutes with two changes. An anti-human IgG alkaline phosphatase conjugate was then added in blocking buffer at a 1/1000 dilution for 1 hour at room temperature on a shaker. After several washes in PBS the colour reaction was developed. A 10X stock of alkaline phosphatase buffer was diluted to 1X strength and to 50mls, 50mg magnesium sulphate heptahydrate (BDH Ltd), 25mg β-naphthal phosphate (Sigma Ltd) and 25mg fast blue BB salt (Sigma Ltd) was then added. The purple colour reaction occurs within seconds.

2.c.2. Characterisation of recombinant GST-p26.

i Propagation of bacterial cultures.

Cultures of *E.Coli* JM101 cells transformed with the HIV-2 p26 recombinant plasmid were propagated overnight in sterile universal containers in an orbital shaking incubator at 37°C in TY-medium containing 100μM ampicillin. Cultures were expanded to 100ml volumes in 250ml baffled culture flasks by diluting 1/20 in fresh medium. Shaking was continued until the cultures reached an OD of 1.0 (600nm) when IPTG was added to a final concentration of 1mM. After a further 2 hours incubation, cells were pelleted in an IEC Centra bench centrifuge at 4000 rpm and resuspended in a 1:50 culture volume of PBS and lysed on ice by sonication for 30 seconds. Triton X-100 was added to 1% (to reduce non-specific binding of bacterial proteins to beads during adsorbtion) and the lysate centrifuged at 12 000rpm to remove insoluble debris. Expression was assessed by SDS-PAGE analysis.

ii. Purification of fusion protein.

Two purification regimes can be applied to yield relatively pure GST fusion proteins.

Batch purification.

One to two millilitres of a 50% suspension (v/v) of glutathione-agarose beads (sulphur linkage, Sigma Ltd) was added to aliquots of the clarified lysate and mixed on a shaker

for 3-5 minutes. The beads were collected by centrifugation and washed 3 times with PBS. Fusion protein was eluted by competition with free glutathione using two consecutive two minute washes with a one bed volume of 50mM Tris-HCl, pH 8 containing 5mM reduced glutathione.

Column chromatography.

Alternatively, glutathione-agarose can be packed into a 15 cm column (Omnifit Ltd) linked to a peristaltic pump, spectrophotometer and chart-recorder (LKB/Pharmacia Ltd). A 10cm bed volume was equilibrated with PBS at a flow rate of 1ml per minute. Clarified lysate was added at a rate of 0.2-0.25 mls per minute and PBS run through at this rate until the baseline values were recorded. Elution buffer containing the free glutathione was then run through the column at the same flow rate and fractions containing the eluted protein collected and pooled. Typically, 20mls of elution buffer was sufficient to displace the fusion protein. Glutathione-agarose beads have a capacity of approximately 8mg of fusion protein per millilitre of swollen beads and were re-cycled periodically by washing in 0.1M sodium acetate, 0.5M NaCl pH4 and then in 0.1M Tris-HCl, pH 7.4 followed by re-equilibration with 50mM Tris-HCl, pH 7.2 or PBS.

iii. Enzymatic cleavage of GST-p26 using Factor Xa.

The eluted peak was pooled, desalted into cleavage buffer (50mM Tris-HCl, pH 8.0, 100mM NaCl, 1mM $CaCl_2$) using a PD10 gel filtration column (Pharmacia Ltd) and cleaved by incubation with the restriction protease Factor Xa (Genex Biotech Ltd, Denmark) at an enzyme-substrate ratio of 1:20 for 5h at 25°C. Following cleavage, the reaction mix was re-applied to the glutatathione column to remove GST, by elution with PBS. The concentration of purified p26 was estimated by spectrophotometry as previously described (Smith and Johnson, 1988). Purified proteins were stored aliquoted at -70°C.

iv. Hyperimmune p26 rabbit antiserum.

A polvalent p26 antiserum was produced using recombinant p26 as the immunogen. A rabbit was inoculated intramuscularly with three injections of recombinant p26 (500μg) in the presence of Freunds complete adjuvant at 6 week intervals and boosted after three

months, performed with the assistance of Dr. C. Loveday, Department of Virology, UCLMS. Antiserum was tested for anti-p26 reactivity using HIV-2 Western blot strips, using a goat anti-rabbit antibody conjugated to horse radish peroxidase for detection of rabbit-specific IgG. Antiserum was stored aliquoted at -20°C.

2.c.3. HIV-2 antigen capture assay.

i. Assay protocol.

A simple antigen capture assay was established as shown in Figure 2.7. Microwells (Nunc Ltd) were coated with the γ-globulin fraction of a high titre positive serum (T1623, B-25) without further purification at a concentration of 5-10μg/ml in 10mM Tris-HCl, pH 7.6 for 12-36 hours and wells quenched with 10mM Tris-HCl, pH 7.6 containing 0.2% BSA. This served as a capture antibody for 90μl aliquots of each tissue-culture supernatant fluid which were incubated at 37°C overnight with 10μl 100mM EDTA (final concentration of 10mM). After washing with piperazine buffer, 100μl of an HIV-2 human serum (DJ) conjugated to alkaline phosphatase was added and further incubated at 37°C for 2-3 hours. After a final washing step, 100μl of substrate (NADP) was added for a further 55 minutes. Catalysis of the dephosphorylation of NADPH to NADH occurs in direct proportion to the amount of bound conjugate (Figure 2.7.). Addition of 50μl of amplifier for 5-10 minutes was followed by 50μl 2M H_2SO_4 to stop the reaction. Plates were read at a wavelength of 492nm. The alkaline phosphatase conjugate was kindly prepared by Clive Dobson, Murex Diagnostics in a lyophilised form.

ii. Measurement of reverse transcriptase in viral cultures.

Three previously established isolates of HIV-2 (LAV-2_{ROD}, CBL-20 and CBL-22) and HIV-1_{CBL-1} were cultivated in C8166 cells in 25cm² culture flasks. The C8166 cell line was chosen since virus is produced rapidly, indicated by a characteristic cytopathic effect. A time-course series of parallel cultures were established for each virus isolate by transferring a small aliquot (500μl) of supernatant to flasks containing uninfected cells. These were maintained over a period of nine days and supernatants (500μl) collected at 0,1,2,3,4,5,7,8 and 9 days. Each aliquot was stored in liquid nitrogen and assayed

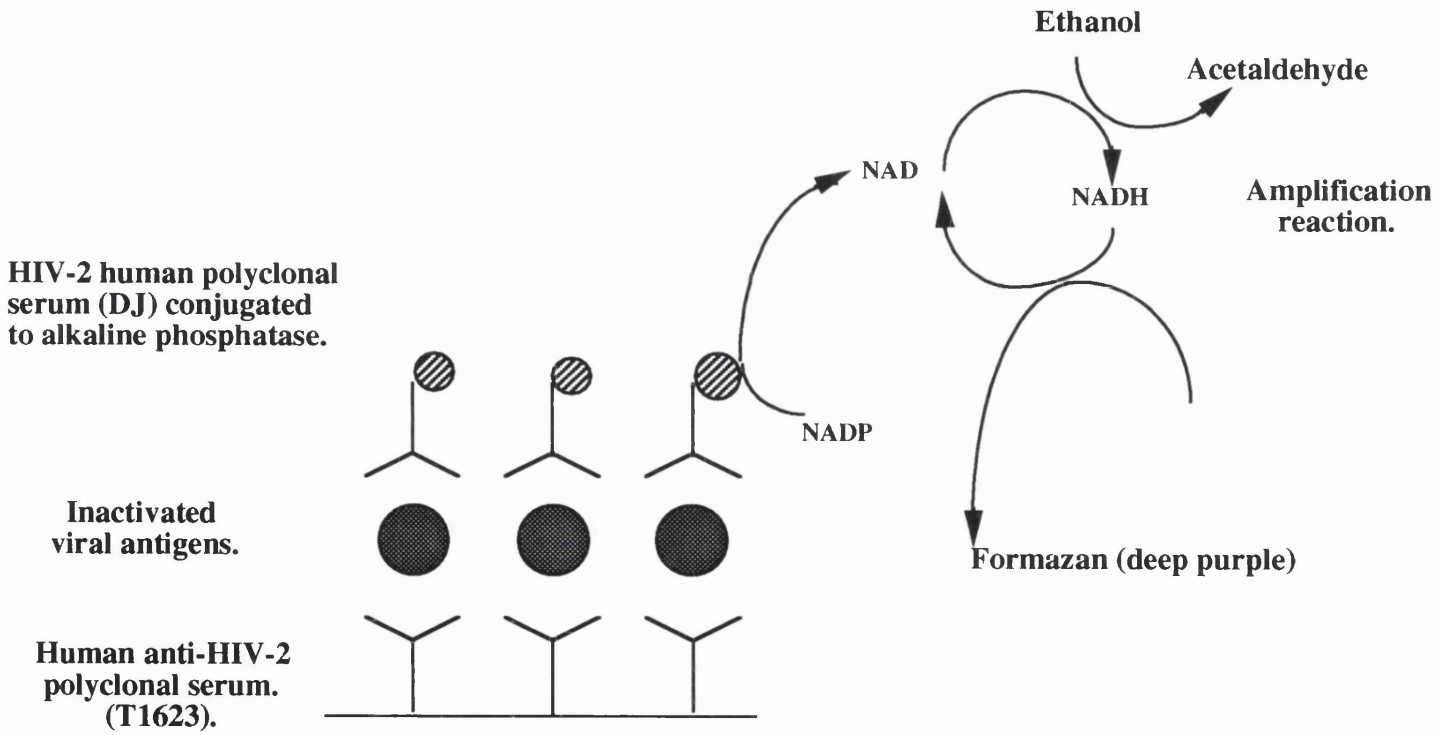


Figure 2.7. Assay for detection of HIV-2 viral antigens in culture fluids. DJ serum directly conjugated to alkaline phosphatase was used to generate the amplification reaction.

together for reverse transcriptase enzymatic activity as a definitive measure and baseline marker of retroviral activity. These were performed by Clive Patience, Chester Beatty Laboratories and were used to evaluate reactivity of the antigen assay.

A commercially available antigen assay (Innotest, Innogenetics Ltd) was also compared with reverse transcriptase levels and performed according to the manufacturers' instructions. This assay is of similar format to the HIV-2 antigen assay developed.

2.c.4. DNA sequencing.

i. Direct solid-phase sequencing protocols.

The p26 sequence of CBL.20-23 was determined using solid-phase sequencing protocols using up to four sequencing primers one of which was used in the original amplification (5' sense) and three additional primers resulting in overlapping sequence fragments of p26 (Figure 2.5.). The 3' antisense primer was re-synthesised for these experiments to include a biotin moiety to allow direct sequencing of single DNA strands. The basic protocol used for p26 was also applied to direct sequencing of the *nef*/U3 LTR region of the HIV-2 genome (Chapter 6). Magnetised streptavidin coated polystyrene particles were used to generate single-strands for direct sequencing as previously described (Hultman *et al*, 1989) as outlined in Figure 2.8. and applied directly to standard DNA dideoxy sequencing procedures (Sanger *et al*, 1977).

Streptavidin-coated dynabeads M-280 (Dynal Ltd), supplied as a suspension at a concentration of 10mg/ml dissolved in PBS pH 7.4, containing 0.1% BSA and 0.02% NaN_3 preservative, were washed in 1X binding and washing buffer (BWB, Appendix 1) in a 1.5ml conical 'Sarstedt' tube using a magnetic particle concentrator. Beads were resuspended to their original volume in 1X BWB. For each sequencing reaction, 20µl (200µg) of washed beads were combined with 10-20µl of biotinylated second round PCR product and incubated at room temperature with the prewashed beads for 15 minutes with continuous agitation. The DNA duplex was melted by addition of 50µl of freshly prepared 0.15M NaOH and incubated at room temperature for a further 10

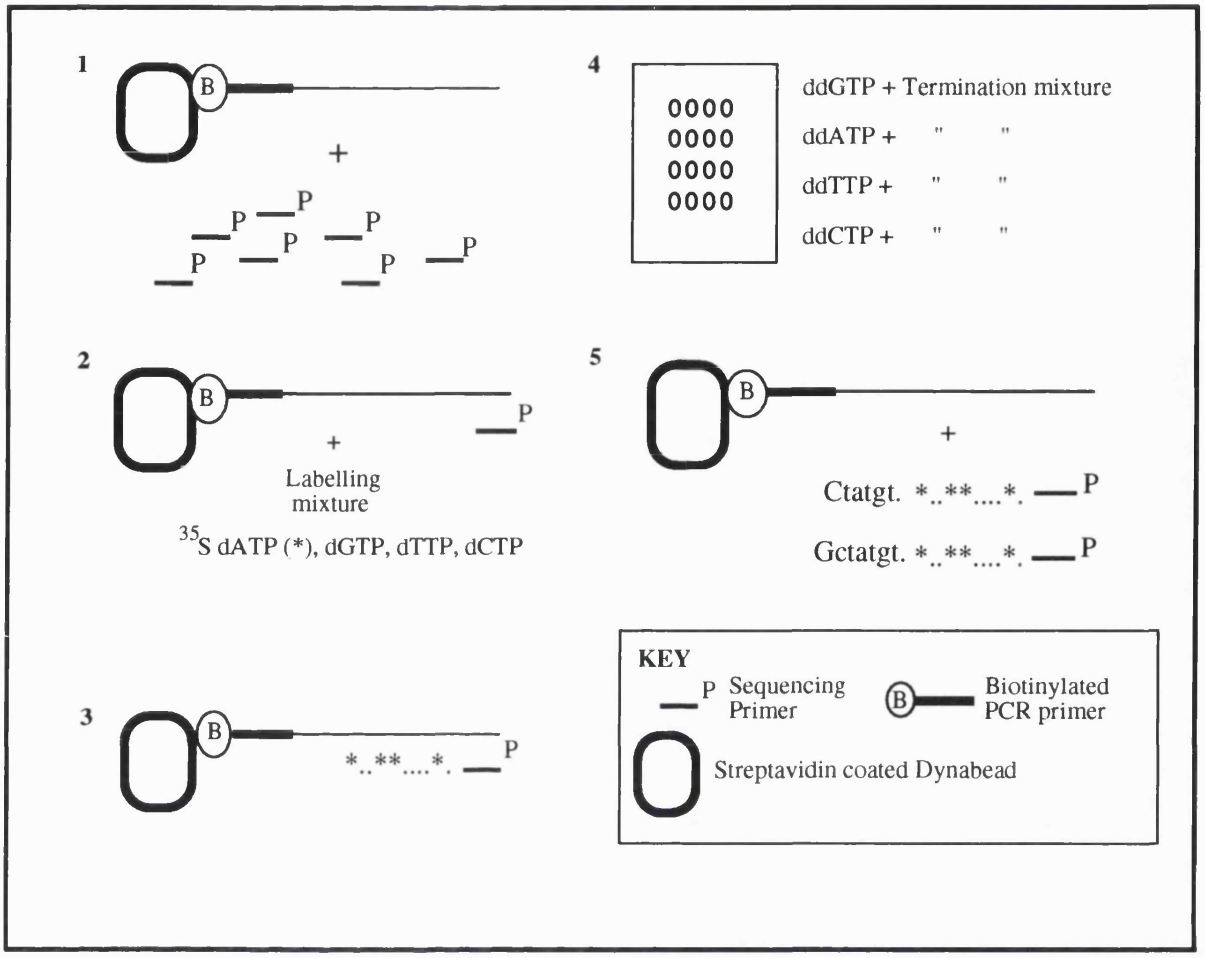


Figure 2.8. Direct solid-phase DNA sequencing of PCR products by the dideoxy chain termination method.

1 Single-stranded DNA is generated utilising biotiylated primers (either sense or antisense) utilising streptavidin-coated magnetised latex microspheres (M280 Dynabeads TM). Oligonucleotide primers which are complementary to the template sequence anneal to the single stranded PCR product.

2 The primer-template is labelled by the incorporation of a radiolabelled nucleotide, in this case ^{35}S dATP.

3 Primers are extended to give a mixture of oligonucleotides of varying lengths.

4 The newly synthesised chains terminate when a ddNTP (dideoxy NTP) is incorporated in place of the corresponding dNTP.The products of the reaction are a series of oligonucleotides the length of which is determined by the distance between the primer and the site of premature termination. Populations of oligonucleotides are generated which terminate at positions occupied by every nucleotide in the sequence.

5 Reaction products are resolved by electrophoresis under conditions which allow discrimination between oligonucleotides which differ in length by only one nucleotide.

minutes. The resulting single-stranded template was washed with 40µl of TE and finally in 10µl of distilled water.

Sequencing reactions were performed in 96 well microtitre plates on a DNA thermal cycling machine with 7µl of immobilised DNA template, 2 pmoles (1µl) of the appropriate sequencing primer and 2µl of 5X sequenase buffer (200mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250mM NaCl). Annealing mixture was heated to 65°C for 2 minutes and allowed to cool to room temperature (below 30°C). A labelling reaction was set up adding 2µl labelling mix (1/30 in distilled water) to the annealed template-primer in the presence of 1µl of 0.1M DTT, 0.5µl [^{35}S] dATP (1400Ci/mmol), and finally, 2µl of Sequenase (Version 2) previously diluted 1/8 in ice-cold TE buffer. This was mixed thoroughly and incubated at room temperature for 2-5 minutes. Termination reactions were completed by pre-warming 2.5µl of each termination mix (ddATP, ddGTP, ddCTP and ddTTP) at 37°C for 1 minute and 3.5µl of the labelling reaction mix when complete added to each and the incubation continued for a further 3-5 minutes. Reactions were stopped by the addition of 4µl of stop solution containing deionised formamide in 10mM TE, pH 7.5, 0.3% (w/v) xylene cyanol and 0.3% (w/v) bromophenol blue. Reactions were stored at -20°C for up to several weeks, or run immediately on a sequencing gel.

Sequenced products were separated using a 0.4mm, 8% denaturing polyacylamide gel prepared using Sequagel reagents (National Diagnostics/Flowgen Ltd). After 3 minutes incubation on a hot-block (>80°C) 3.5µl aliquots of sequenced products were loaded onto the gel and electrophoresed on a vertical sequencing apparatus (BRL Ltd) for 2-4 hours using a constant power supply unit (Pharmacia Ltd) at 65 watts. Sodium acetate (40g) was added to the lower chamber after 1.5-2 hours and electrophoresis continued. Gels were fixed in a solution of 5% acetic acid (v/v) and 5% methanol (v/v) for two 15-minute intervals and dried under vacuum at a temperature of 80°C for 60-90minutes on a slab gel-dryer (BioRad Ltd) on Whatmann 3MM chromatographic filter paper. Dried gels were subjected to autoradiography at room temperature using Fast Blue X-ray film (GRI Ltd) and developed at varying intervals (overnight to several weeks) using an automatic X-ray developer (GRI Ltd).

ii. Phylogenetic analyses.

The phylogenetic relationship of the p26 sequences (CBL.20-23) generated were compared to HIV-2_{ROD} by the maximum likelihood method (Swafford and Olsen, 1990). Briefly, this method seeks to infer the history that is most consistent with an observed data set, where the data are the observed nucleotide sequences and the unknown parameters are the branching order and the tree branch lengths. Computer analyses were applied by Dr. P. Balfe. Division of Virology, UCLMS, using the DNAML programme (Felsenstein,1988).

Chapter 3.

Detection of HIV-2 antibody (anti-HIV-2).

3.a. Introduction.

Both HIV-1 and HIV-2 elicit a similar antibody response. Seroconversion usually occurs within several weeks following primary infection and results in virus-specific antibodies detectable in the serum. Comparison of antibody profiles in HIV-1 and HIV-2-infected individuals indicates that both type-specific and cross-reactive antibodies are produced. Examination of the neutralisation properties of human sera have indicated that HIV-2 induces antibodies that are able to cross-neutralise HIV-1 although HIV-1 antibodies are more group specific and have little neutralising effect on HIV-2 (Weiss *et al*, 1988b). The detection of HIV-2-specific antibody (anti-HIV-2) has been used as a marker of infection and a number of different approaches have been applied for this purpose.

The use of the radioimmunoprecipitation assay (RIPA) was important in initially distinguishing HIV-2 from HIV-1, linking it more closely to SIV than to HIV-1 (Clavel *et al*, 1986a). RIPAs have not been generally applied to the diagnosis of HIV-2 infection since the need to culture infected cells is a severe limitation of this approach. Modifications of the standard RIPA procedure have been proposed for HIV-2, however, utilising iodinated antigens consisting of virus lysate spiked with recombinant HIV-2 gp105 to increase sensitivity for diagnostic purposes (Selvam *et al*, 1993). This technique remains cumbersome, however, and has not gained widespread use as a diagnostic tool. Western blots, however, have been commonly used for the detection of anti-HIV and have become integrated into algorithms used for the diagnosis of HIV infection by serological means and commonly applied in confirmatory testing protocols. Strong cross-reactions with HIV-1 and HIV-2 sera with the heterologous viral antigens in Western blots have been demonstrated, however, making diagnosis of the infecting virus difficult in these circumstances (Tedder *et al*, 1988; De Cock *et al*, 1991).

Immunoassays in a microtitre format have also been commonly applied for the detection of HIV-specific antibody and were initially based upon culture-derived viral antigens.

Four main types of immunoassay may be employed for the detection of anti-HIV (Tedder, 1986; Cameron, 1987) referred to as type 1: antiglobulin, type 2: competitive, type 3: reverse capture and type 4: immunometric (Figure 3.1). Culture-derived viral antigens, recombinant polypeptides and synthetic oligopeptides have been applied in different assay formats. Highly immunodominant domains of the HIV-1 transmembrane glycoprotein (TMP, gp41) have been identified (Ho *et al*, 1987; Modrow *et al*, 1987) and applied in site-directed serological assays. In HIV-2 and SIV strains grown *in vitro*, the analogous protein appears to have a smaller molecular weight of approximately 36 kDa (gp36) due to a premature stop codon in the *env* gene causing the truncated form of the transmembrane glycoprotein, although native SIV strains carry a full-length gp41 (Kodama *et al*, 1989, Cranage *et al*, 1989).

The targetting of the TMP as an effective means for identifying HIV-2-related lentiviruses was first described by Norrby *et al*, (1987). This site-directed approach to HIV serodiagnosis is now widely employed and this region has been used for the differentiation of HIV-1 from HIV-2. Although screening assays which detect both anti-HIV-1 and anti-HIV-2 are now in widespread use, it is also important to confirm the specific reactivity using type-specific assays to determine which is the infecting virus. Investigation of the specificity of the anti-HIV response in certain geographical areas increases the need for highly specific assays and therefore assay format becomes particularly important.

The competitive format for serological testing has been demonstrated as an effective means of detecting virus-specific antibody even when quite crude preparations of antigen have been employed, notably for the detection of anti-HTLV-1 and anti-HTLV-2 (Tedder *et al*, 1984). Following the isolation of HIV-1 (LAV/HTLV III), a competitive EIA for detection of anti-HIV was established initially using antigens extracted from cultured HIV strains (CBL-1) and using human polyclonal antisera obtained from HIV-infected individuals. A number of solid-phases were employed during the evolution of the competitive anti-HIV-1 assay including from the capture of culture-derived antigens onto the solid-phase by polyclonal sera from HIV-1-infected patients, which were subsequently replaced by anti-*gag* (p24) monoclonal antibodies (Ferns *et al*, 1988). The

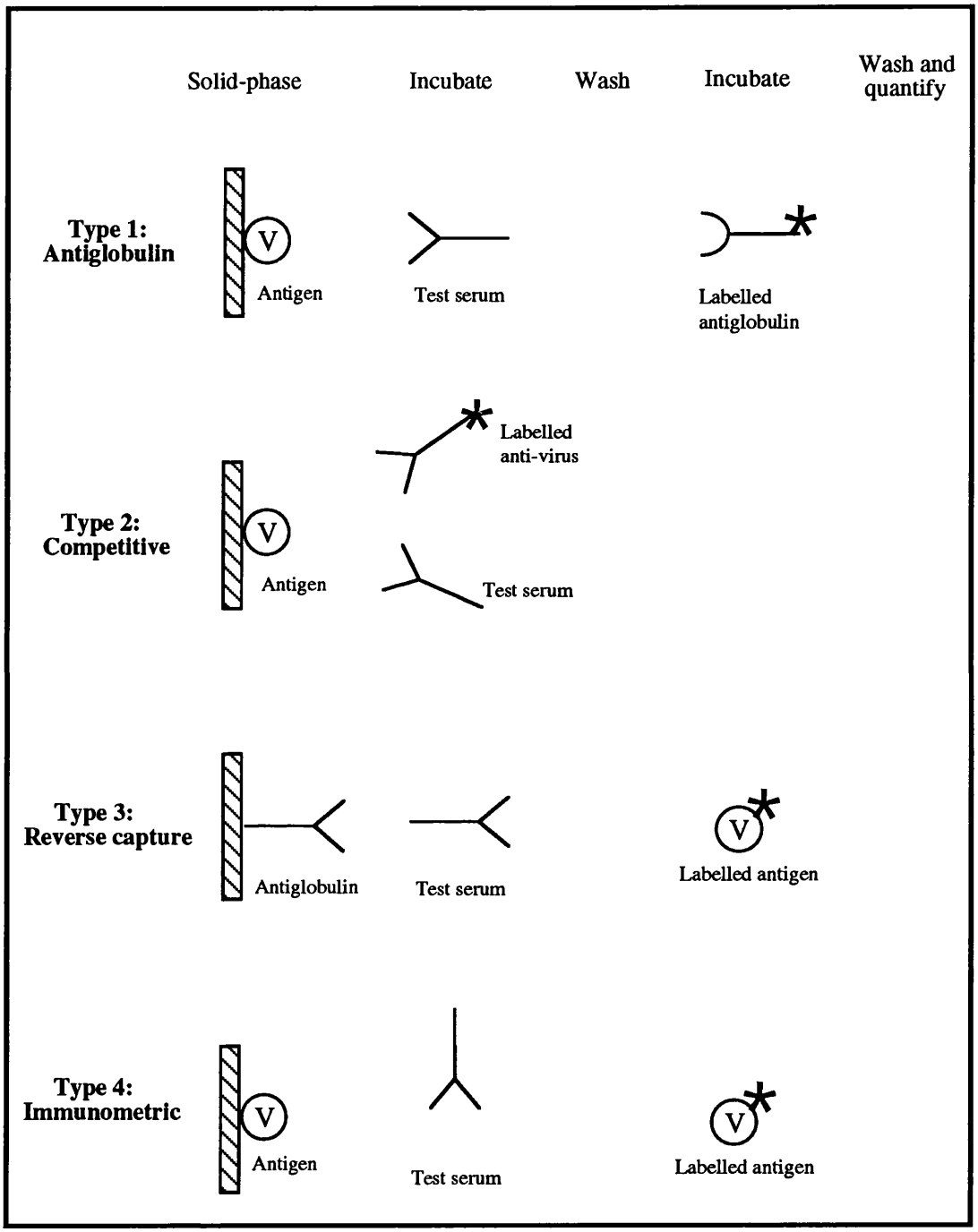


Figure 3.1. Four formats used in serological assays for the detection of anti-HIV.

assay which is commercially available consists of an anti-*gag* monoclonal antibody acting as a capture reagent for a recombinant *gag-env* fusion protein derived by expression in *E.Coli.*

An HIV-2 competitive assay was subsequently established firstly in the Department of Virology using LAV-2_{ROD} as the viral antigen and later with Gambian HIV-2 strains, in particular the CBL-20 isolate (Schulz *et al*, 1990). The supply of this assay to the MRC Unit in The Gambia has been important in establishing the prevalence of HIV-2 infection in The Gambia. The substitution of a recombinant polypeptide in place of culture-derived antigens in a competitive format is described and applied to the diagnosis of possible dual infections and for identifying related non-human primate lentiviruses.

3.b. Results.

3.b.1. Results of field studies with VC-EIA using different panels of sera.

The results of each of the panels of sera used to evaluate the competitive EIA based on the viral lysate antigen (VC-EIA) in The Gambia are described including results obtained with the ELAVIA-2 and Western blot assays used in the evaluation. These results therefore represent a comparison of an antiglobulin assay (ELAVIA-2) with a competitive assay (VC-EIA), both of which are based on culture-derived viral antigens.

i. *Panel 1*: Seroprevalence of HIV-2 in a rural population of The Gambia.

The results of screening for HIV-2 infection in the general population of a rural area of The Gambia, comprising 491 randomly chosen adults, indicated a prevalence of 1.02% (5/491) when concordant competitive EIA and ELAVIA-2 results were used (Table 3.1). When only the competitive assay was used the prevalence was estimated to be 1.8% (9/491) but if sole use of the ELAVIA-2 assay was made, this estimate rose to 6.9% (34/491).

The 29 sera which were positive by ELAVIA-2 but negative by VC-EIA were also tested by HIV-2 Western blot and 28 showed an indeterminate pattern characterised by bands to either *gag* or *pol* gene products with no *env* bands; the other was unreactive. Of the five sera concordantly positive by EIA four were positive by Western blot; the other which was weakly positive by both the competitive assay and by ELAVIA-2 showed, however, an indeterminate Western blot pattern. Thus, HIV-2 seropositivity was determined as 0.8% and represents an estimate of the seroprevalence in this particular study population at the time of the study. Fifty sera negative by both EIAs were also tested by Western blot and all were negative.

ii. *Panel 2*: Reproducibility of the VC-EIA.

Sera from 250 prostitutes of whom 51 were HIV-2 seropositive as determined by Western blot, were examined twice under code to investigate the reproducibility of the results of the competitive assay (Table 3.2). Two of the 189 (1.06%) negative in the first test were classified as positive in the second test. Three of 61 (4.9%) sera classified as positive on the first test were found to be negative on the second test. None of these five

Assay	ELAVIA-2			
HIV-2 competitive (VC-EIA)	Result	Negative	Normalised optical density >1	Total
	Negative	453	29	482
	Normalised optical density >1	4	5	9
	Total	457	34	491

Table 3.1. Reactivity of 491 sera from Gambian patients tested by ELAVIA-2 and VC-EIA.
An initial seroprevalence of 1.02% (5/491) was determined based on five concordantly reactive sera. Four of these five were also confirmed by Western blot and a revised seroprevalence of 0.8% (4/491) recorded.

	First screen				
	Normalised optical density	<1	>1<5	>5	Total
Second screen	<1	187	2	1	190
	>1<5	2	5	1	8
	>5	0	1	51	52
	Total	189	8	53	250

Table 3.2. Reproducibility of the anti-HIV-2 VC-EIA determined by testing 250 sera twice under code.

misclassified sera were positive on Western blot. All of the 51 sera which were positive by Western blot were classified as strongly positive on both tests. With any assay, the criteria for positivity may be subject to scrutiny and clearly greater security may be placed on test results which are either strongly reactive (>5) or strongly negative (<1) on two separate occassions. Where antibody reactivity is weak, but clearly significant (>1<5) so less certaintly may be placed on such values although where this reactivity is consistent further investigation will be required. Eight samples out of 250 tested fell into this latter category with five falling into this intermediate category of positivity when tested twice.

iii. *Panel 3*: Comparison of results of screening tests and Western blot.

Four hundred and eighty selected sera from surveys of the general population, patients and individuals in high risk groups including prostitutes and STD patients were tested by ELAVIA-2, VC-EIA and with an HIV-2 Western blot (Table 3.3.). From the total of the 480 sera, 304 (63.3%) were positive in the competitive assay; of these 259 (85.2%) proved to be positive on Western blot. In comparison, 410 sera (85.4%) were positive on the ELAVIA-2 assay; of these 258 (62.9%) were positive on Western blot. The two assays differed markedly overall with sera exhibiting weak reactivity (>1<5) and which if genuinely contained anti-HIV-2 would only be at very low levels. A total of 12.7% (61/480) sera tested by competitive EIA fell into this category compared with 32.1% (154/480) tested by ELAVIA-2. This low level of reactivity, at or around the cut-off value for each assay, was therefore more common with the antiglobulin format.

Where sera were weakly positive in one assay (>1<5) but negative in the other assay (<1), the difference in the two assays becomes particularly apparent. A total of 118 sera were weakly reactive in the ELAVIA-2 assay but negative by VC-EIA, none of which were positive by Western blot. This is compared with only 19 sera which were in a similar category when tested by competitive EIA, one of which was also positive by Western blot. The performance of the two assays where antibody reactivity is marginal and therefore most difficult to interpret reveals significantly better specificity by competitive assays.

Assay	ELAVIA-2				
Anti-HIV-2 competitive EIA.	Normalised optical density	<1	>1<5	>5	Total
	<1	50 (0)	118 (0)	8 (0)	176 (0)
	>1<5	19 (1)	17 (4)	25 (12)	61 (17)
	>5	1 (0)	19 (19)	223 (223)	243 (242)
	Total	70 (1)	154 (23)	256 (235)	480 (259)

Table 3.3. Reactivity of 480 sera from the general population, patients attending a clinic for STD, and prostitutes compared by competitive EIA (VC-EIA), ELAVIA-2 and Western blot. Results of Western blots are given in parentheses. Criteria for Western blot positivity are given in the text.

In the case of the strong concordant reactions, however, whereby 223 sera were shown to be strongly reactive in both EIAs, these were all demonstrated to be positive on Western blot. The predictive value of concordant strong reactions was therefore 100% (223/223) with reference to Western blot. The predictive value for strong reactions in each assay separately was 99.6% (242/243) for the competitive VC-EIA and 91.8% (235/256) for ELAVIA-2. By comparison, the sensitivity by which a serum confirmed positive by Western blot and gave a strong reaction was 93.4% and 90.7% for the VC-EIA and ELAVIA-2 respectively. All Western blot confirmed sera were detected by the VC-EIA (259/259) though one serum was not detected by the ELAVIA-2 assay.

3.b.2. Recombinant competitive EIA (RC-EIA).

i. Assay optimisation.

The RC-EIA was optimised using the freeze-dried DJ conjugate since large quantities of this reagent were available. Optimal concentrations of both BG-79 capture antibody and solubilised preparations of recombinant antigen were determined together by cross-titrations summarised in Table 1, Appendix 2. No attempts were made to assess the relative amount of *env* protein expressed due to the large excess of bacterial proteins and the performance of the protein as an antigen was determined empirically according to the specific reactivity with the anti-HIV-2 conjugate. NP-40 reduced the interference of SDS in antigen binding and provided a more evenly coated solid-phase.

Optical density values of less than 100 (OD X1000) for the positive (1/50 dilution) serum and greater than 2000 for the negative (NHS only) were imposed. This provided an initial indication of competitive reactivity with test serum and conjugate binding in the absense of competing antibody. Capture antibody concentrations of 1.25 and 2.5μg/ml were sub-optimal. Although, a coat of 10μg/ml of BG-79 decreased the ability of the monoclonal antibody to act as an efficient capture antibody strong binding was demonstrated at this concentration. However, a concentration of 5μg/ml of BG-79 capture antibody provided acceptable values with a range of antigen dilutions and was used in all further experiments to determine the relative amount of antigen required for solid-phase coating. This was performed empirically for each antigen batch, as illustrated

in shown in Figure 3.2. where the optical density for each serum dilution of DJ is compared. A serum dilution of 1/3000 represents an approximate 50% inhibition and cut-off value and therefore an antigen dilution of 1/5000 provided both a relatively sensitive assay with a wide range of optical density values. Although sensitivity obtained in such experiments appeared to be quite good, the reactivity of DJ in the RC-EIA appeared to be less than in the VC-EIA and 50µl of serum was used rather than 25µl in the VC-EIA. The relative sensitivity of the HIV-2 competitive assays to other assays was therefore investigated further.

ii. Sensitivity of VC-and RC-EIAs.

Four sequential sera containing low antibody levels obtained from an individual (patient G003) who was presumed to be seroconverting were initially used to compare the VC-EIA with the ELAVIA-2 assay. Interpolated end-point values were obtained for each of the six assays for patient G003 (Figure 3.3). The rise in antibody levels in each assay indicates that these sera were most probably from an individual who had recently acquired HIV-2 infection. It is interesting to note that for five of these assays, all except the VC-EIA assay, the first sample would be classified as indeterminate or negative according to the manufacturers' criteria for a positive antibody reaction. The VC-EIA records an interpolated end-point titre of 25 which increases to 500 in the second sample. It is unfortunate that no time-points were available for these samples although for comparative purposes of the assays this is not necessarily important. The VC-EIA was demonstrated to have good reactivity with these low-level antibody sera indicating that competitive assays can be equally as sensitive, if not more so, that other assay formats. Of the commercially available assays only the ELAVIA-2 assay, designed specifically for anti-HIV-2 detection only, and the Wellcozyme 1+2 assay which is immunometric in format provide comparable responses. The ELAVIA-2 Rapid Mixt, Abbott recombinant and Enzygnost combination assays all yielded poor HIV-2-specific responses with these samples.

These studies were extended to 15 sera for which the interpolated end-point titres were obtained for each of the above assays. The findings of this set of data are shown in Table 2, Appendix 2. indicating that the most sensitive assay was the VC-EIA which was on

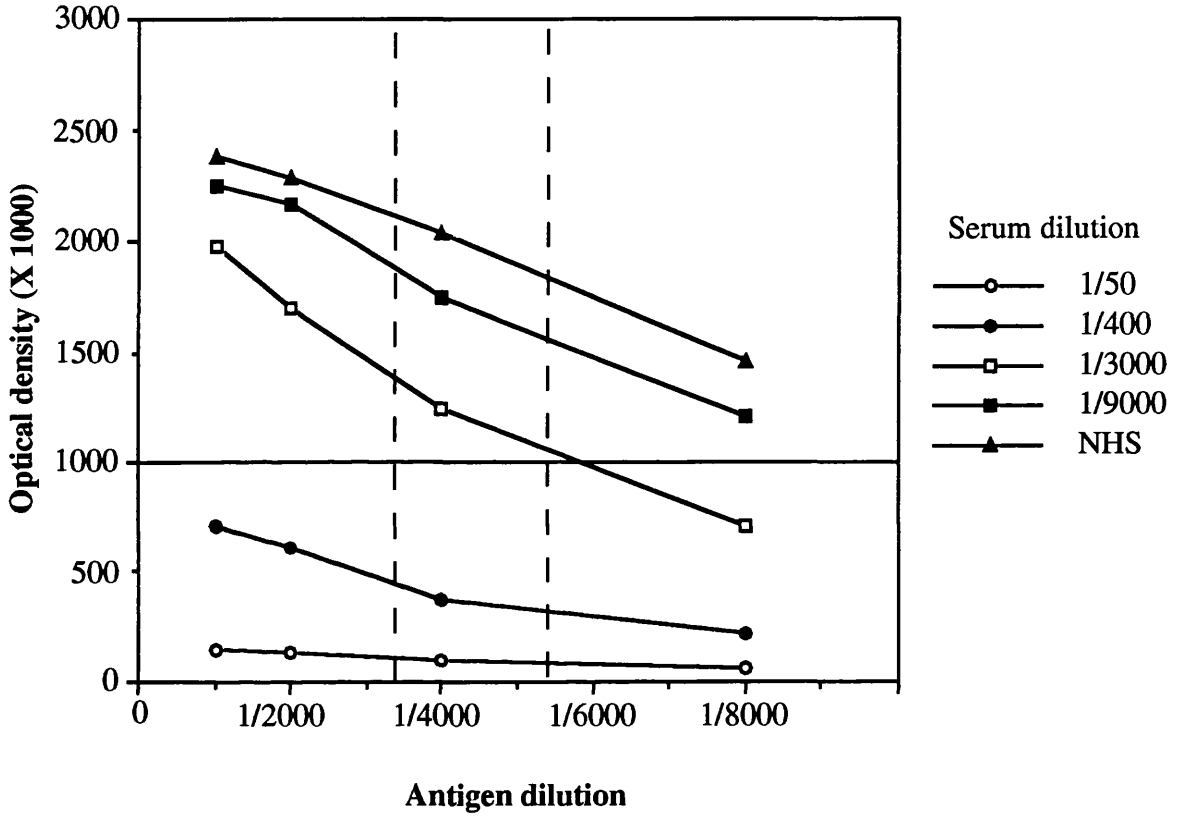


Figure 3.2. Titration of different dilutions of DJ serum with varying concentrations of antigen. Titrations were performed with BG-79 coated at 5µg/ml. Horizontal line indicates a theoretical 50% end-point with the vertical dashed lines corresponding to the range of antigen dilution giving the widest dynamic range.

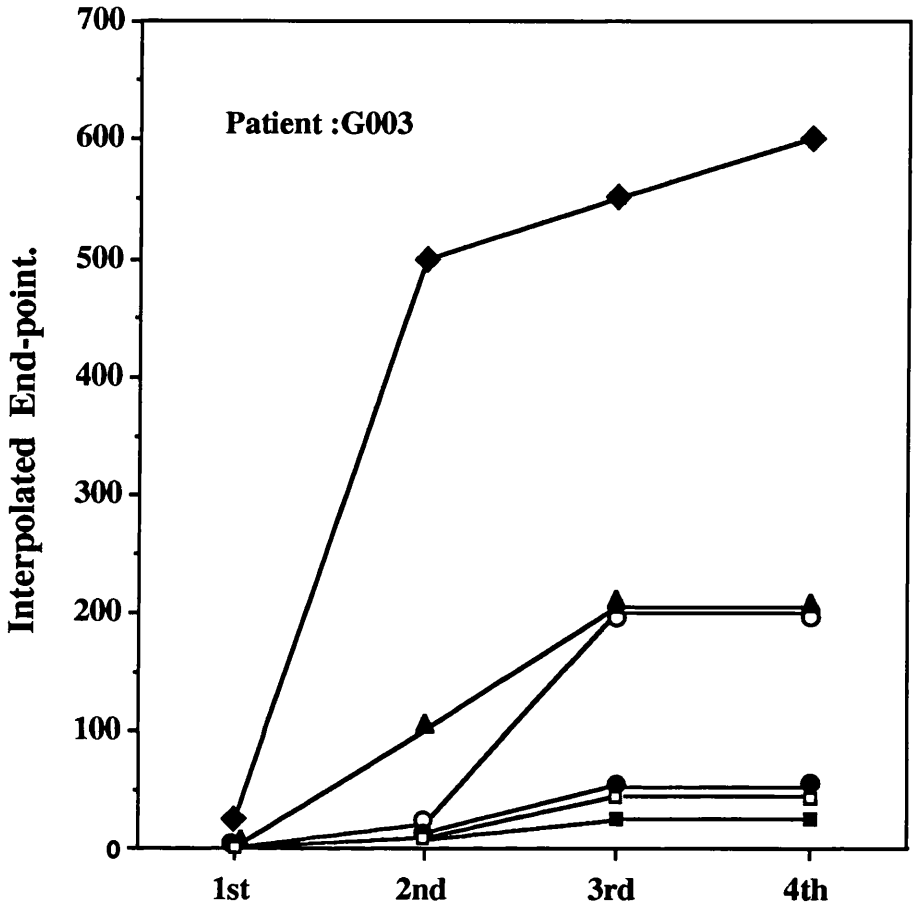


Figure 3.3. Anti-HIV-2 reactivity of four consecutive sera from a single donor recently infected with HIV-2. Interpolated end-points were obtained with six assays with an HIV-2 component.

Competitive EIA for anti-HIV-2 (UCLMS)

ELAVIA-2 (Diagnostics Pasteur)

Wellcozyme combined 1+2 (Murex Diagnostics)

ELAVIA Rapid Mixt (Diagnostics Pasteur)

Abbott recombinant 1+2 (Abbott Diagnostics)

Enzygnost 1+2 (Behring Diagnostics)

average able to detect end-point samples two-fold greater than the ELAVIA-2 assay. Although none of the combined assays approached the sensitivity of the VC-EIA, all detected anti-HIV-2 sera with reasonable proficiency.

Although the HIV-2 RC-EIA had not been fully evaluated in West Africa, it was shown to perform well on a panel of Gambian sera tested in the UK. A small number of sera (37) which signalled strongly in the VC-EIA were also tested in the RC-EIA and were shown to exhibit similar reactivities when tested undiluted. Sixteen of these sera, including the 2nd, 3rd and 4th seroconversion samples, were titrated in NHS using a ten-fold dilution series across their end-point in both VC-EIA and RC-EIA and compared by log-log plots (Figure 3.4.).

The general shift in antibody titres away from their origin and towards the viral lysate antigen indicates a reduction in overall sensitivity of the RC-EIA compared to the VC-EIA of between two to three-fold. This is particularly reflected in the seroconversion samples which had higher end-point titres in the VC-EIA. The results in the RC-EIA, in terms of end-point sensitivity, were therefore comparable to the results obtained with the ELAVIA-2 assay.

iii. Reactivity of anti-HIV-1 in HIV-2 competitive assays.

To investigate the reactivity of sera containing anti-HIV-1 in both VC-EIA and RC-EIAs, ten sera from HIV-1-infected Gambian patients were tested by both the VC-EIA and RC-EIA assays. Standardised optical densities were derived for the 10 Gambian samples whereby a value less than one is taken to be positive and was used to compare reactivities in the two assays. The results are shown in Figure 3.5. The sera tested were found to react in the recombinant assay to a lesser degree compared with the viral lysate antigen since five of the ten sera which were positive in the VC-EIA were only weakly reactive in the RC-EIA. This reduced reactivity may be partly explained by the reduced sensitivity of the RC-EIA although the reactivities of five of the sera were the same in both assays. These cross-reactions, however, were weak and disappeared completely at a dilution of 1/100.

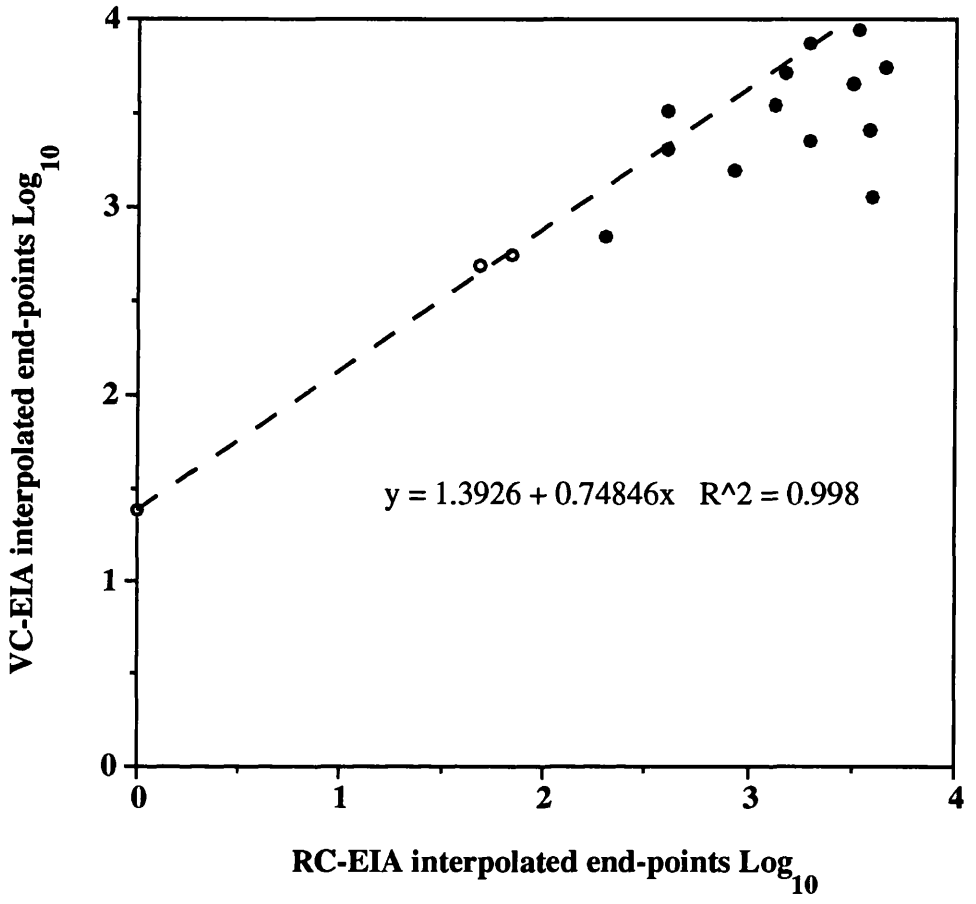


Figure 3.4. Comparison of interpolated end-point titres of sixteen sera containing anti-HIV-2 in VC and R competitive assays. Open circles represent three seroconversion circles. Correlation coefficient is shown whereby the intercept with the y axis indicates the reduced sensitivity of the RC-EIA compared with the VC-EIA.

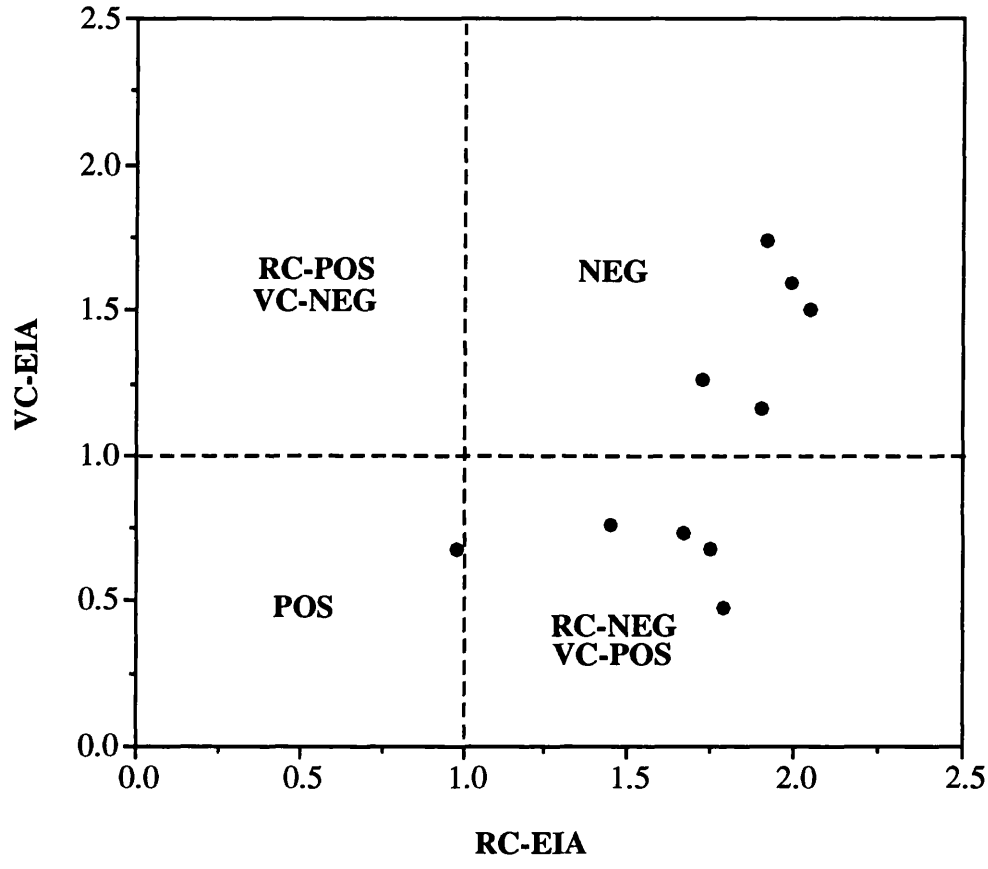


Figure 3.5. Comparison of reactivity of ten Gambian sera containing anti-HIV-1 in the viral lysate (VC) and recombinant competitive (RC) assays. Standardised optical densities were employed whereby dashed lines indicate a cut-off value of 1.0 representing 50% inhibition of the conjugate.

The extent of cross-reacting HIV-1 antibodies was further investigated with 68 UK sera which had previously been tested within the Department of Virology and found to contain HIV-1 antibody by confirmatory testing. These latter samples were selected from HIV-1-infected individuals in London, not The Gambia, since they allowed comparison of larger numbers of sera containing anti-HIV-1 and were also unlikely to contain specific anti-HIV-2 since HIV-2 infection in the UK is rare.

The distribution of the normalised optical densities for 32 samples which had values either near to, or greater than, the cut-off value of 1 is shown in Figure 3.6. For comparisons of the range of reactivity of these sera, normalised optical densities were used. These values, however, may also be expressed as percentage inhibition of the conjugate, and were found to give a percentage inhibition <90% when tested undiluted, with the exception of one sample which gave >90% binding. This also represents a measure of the binding capacity of these sera which ranged from 50-85% inhibition. Although these samples would be viewed as being initially reactive, these would be identified as sera with low specific reactivity. All 32 sera when subsequently re-tested at 1/10 and 1/100 dilutions and the normalised optical densities plotted together (Figure 3.6.) were found to have reactivity below the cut-off value with the exception of one sample, which only became unreactive at a dilution of 1/100. The results of this indicated that although HIV-1 sera do cross-react in the recombinant competitive HIV-2 assay, it is mostly weak and disappears when sera are diluted 1/10. All HIV-2 sera studied, including those with low antibody levels by end-point titration gave very strong inhibition (>90%) either undiluted or at a 1/10 dilution and so would be easily distinguishable from HIV-1 sera.

3.b.3. Applications of HIV-2 competitive assays.

i. Resolution of sera exhibiting strong dual reactivity.

During these studies, however, a small number of sera were identified as having strong reactivity in both HIV-2 competitive assays and the HIV-1 competitive EIA. Sera showing this very strong dual reactivity were investigated further. Titration curves of standardised optical densities were constructed to determine the relative reactivity of ten of these samples in the competitive HIV-1 EIA and the VC-EIA and RC-EIA for anti-

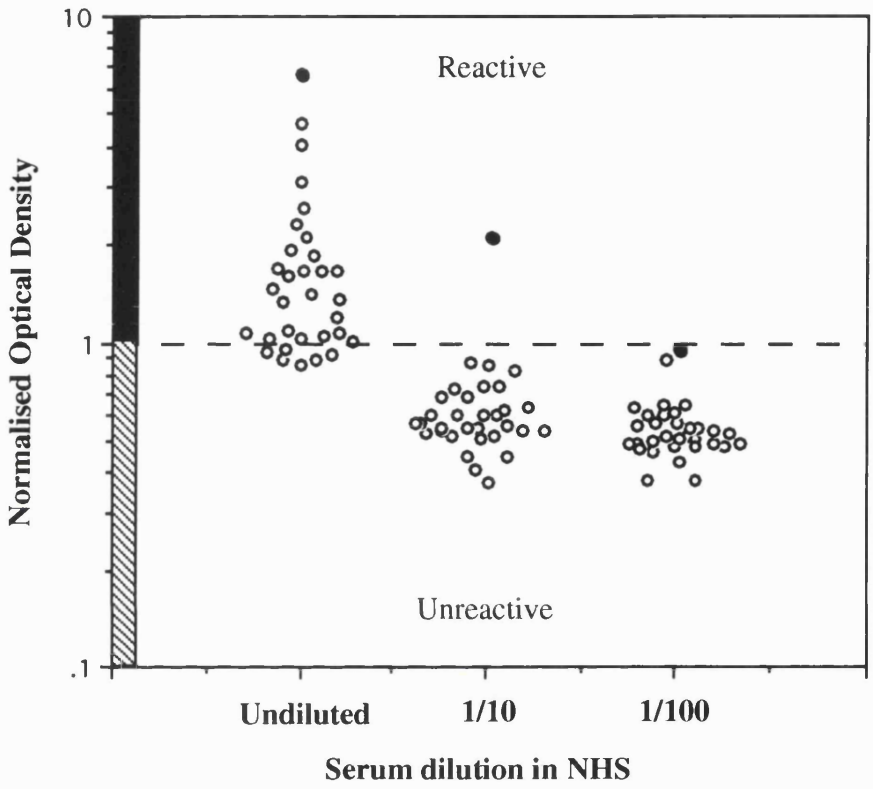


Figure 3.6. Comparison of reactivity of 32 sera containing anti-HIV-1 tested by HIV-2 RC-EIA. Normalised optical densities of >1 indicate a serum classified as reactive. One sample (closed circle) remained reactive at a dilution of 1/10 but was unreactive when tested at 1/100.

HIV-2. The extent of reactivity in each assay was determined and which varied for some sera as shown in Figure 3.7. For example, sample H1650 clearly demonstrated predominantly anti-HIV-1, in contrast to sample I55 which contained anti-HIV-2 only. Whereas for the most part where dual reactive sera were identified they could be easily differentiated from one another at dilutions of 1/10 and 1/100 at which the dominant antibody response would be identified. However, a small number of sera (RE 188, RE 231, H 1891, RE 200, RE 56, H780, RE 16, RE 21) still exhibited strong dual reactivity at higher dilutions and it proved impossible to assign these as either anti-HIV-1 or anti-HIV-2 predominant.

Sera showing the strongest dual reactivity are shown in Figure 3.8. where the regression lines are virtually parallel for each assay including both VC and RC-EIAs. In some situations, it appeared that the relatively lower sensitivity of the RC-EIA may have conferred on it less susceptibility to HIV-1 cross-reactivity, allowing reassignment of sera previously unassigned. RE 200 and H 1650 were therefore re-classified as anti-HIV-1 predominant. However, the phenomenon of strong dually reactive sera was clearly observed even using competitive assays although these represent a very small proportion of sera reactive for either HIV-1 or HIV-2 antibody in the samples tested from The Gambia.

ii. Suitability of alternative HIV-2 conjugates.

The suitability of alternative plasma sources as conjugates was investigated and five other donors were compared. Dilutions of DJ serum of 1/50, 1/400, 1/3000 and NHS were competed against six conjugates (DJ, BM, B-25, IP, GA-20 and GA-21). Dilutions of the stored conjugates kept in 50% v/v in glycerol at -20°C, were made in conjugate diluent (Appendix 1) at dilutions of 1/250, 1/500, 1/1000 and 1/1300. Actual optical densities (X1000) were compared to assess the relative performance of each conjugate. The reactivity of each of these conjugates was found to be broadly similar as shown in Figure 3.9. with the exception of donor IP which reacted poorly. All the others displayed strong binding in the absence of competing antibody and showed strong inhibition of dilutions of HIV-2 antibody. These data suggest that most plasma/serum samples derived

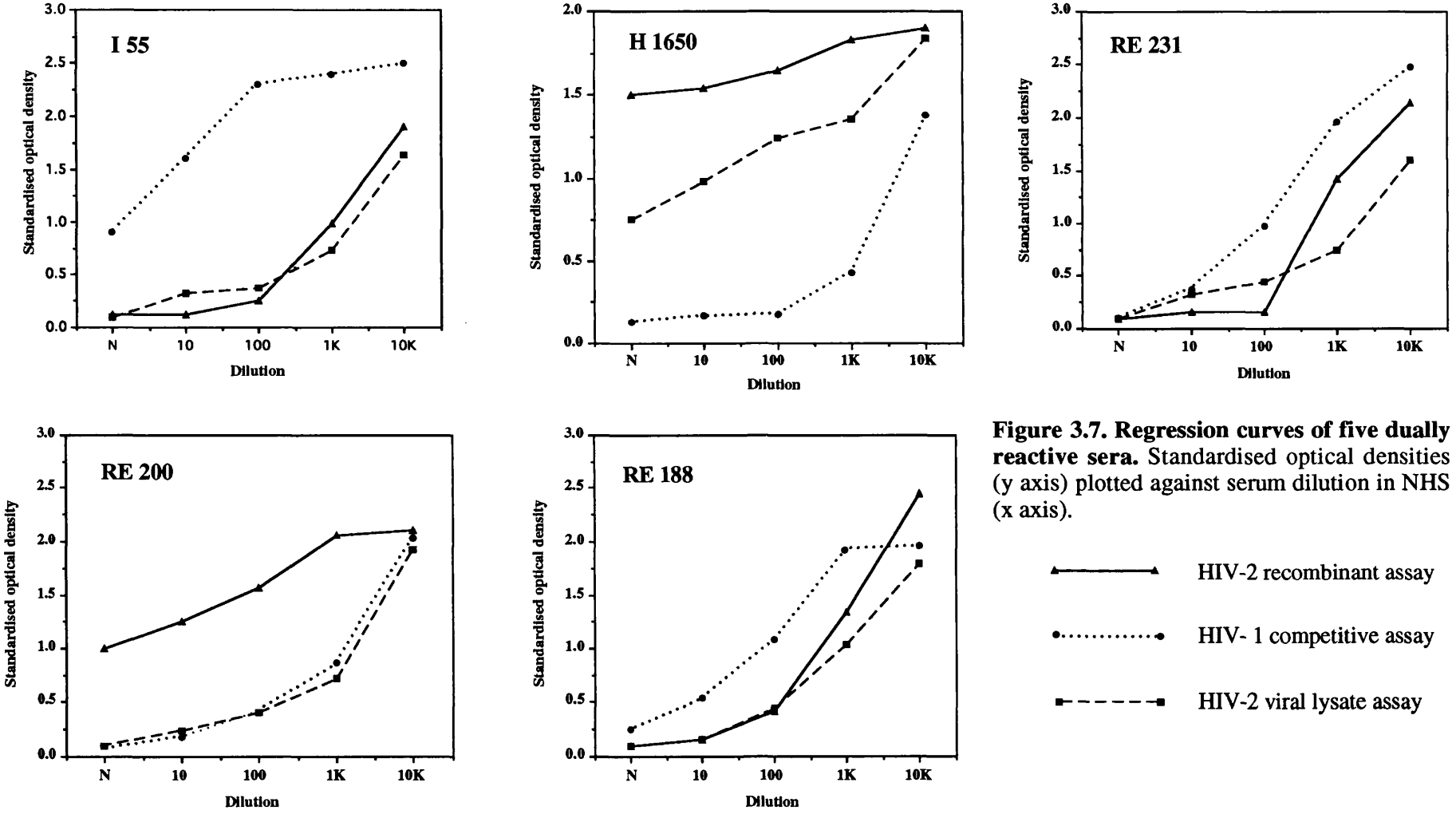


Figure 3.7. Regression curves of five dually reactive sera. Standardised optical densities (y axis) plotted against serum dilution in NHS (x axis).

HIV-2 recombinant assay

HIV- 1 competitive assay

HIV-2 viral lysate assay

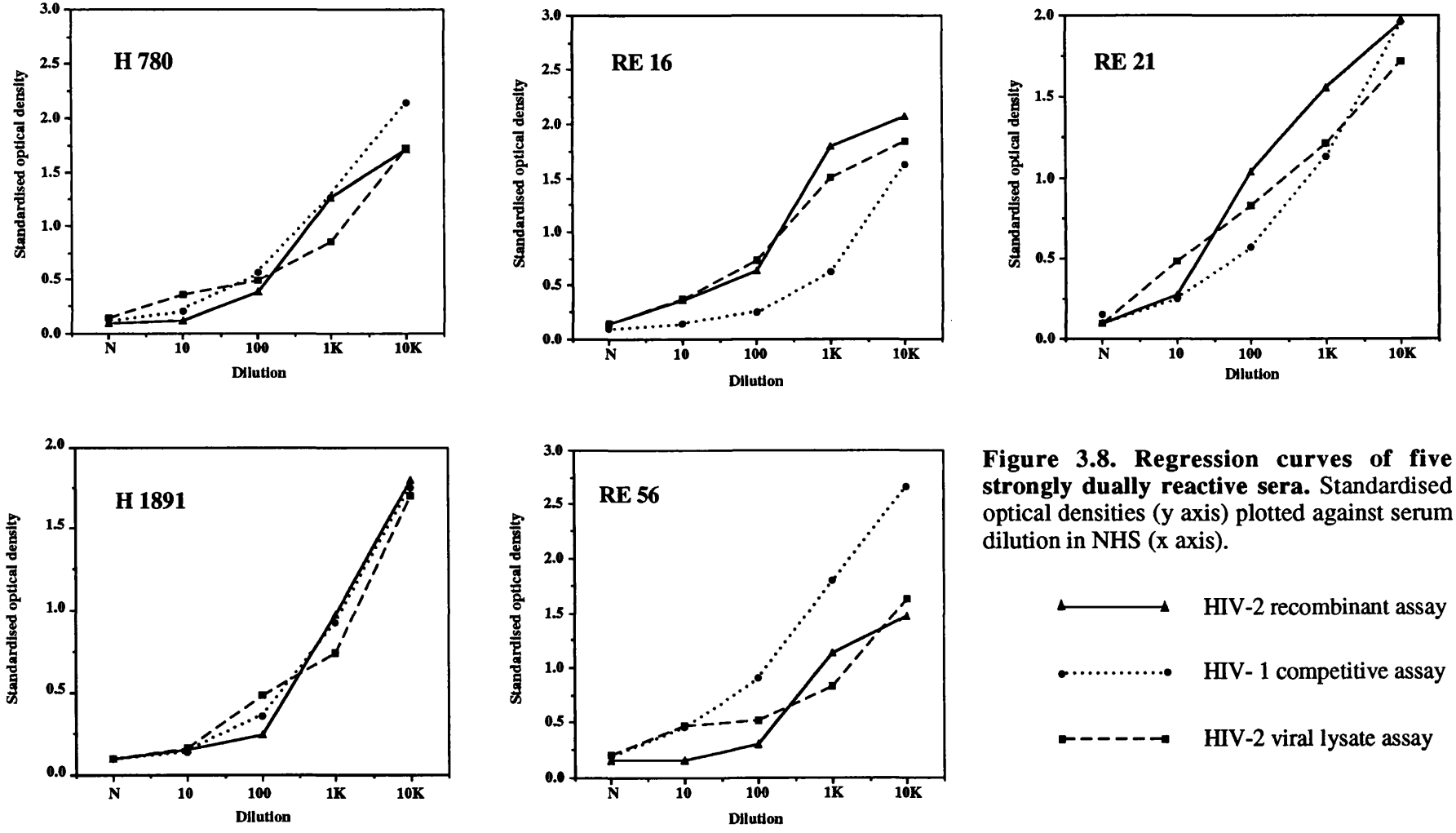


Figure 3.8. Regression curves of five strongly dually reactive sera. Standardised optical densities (y axis) plotted against serum dilution in NHS (x axis).

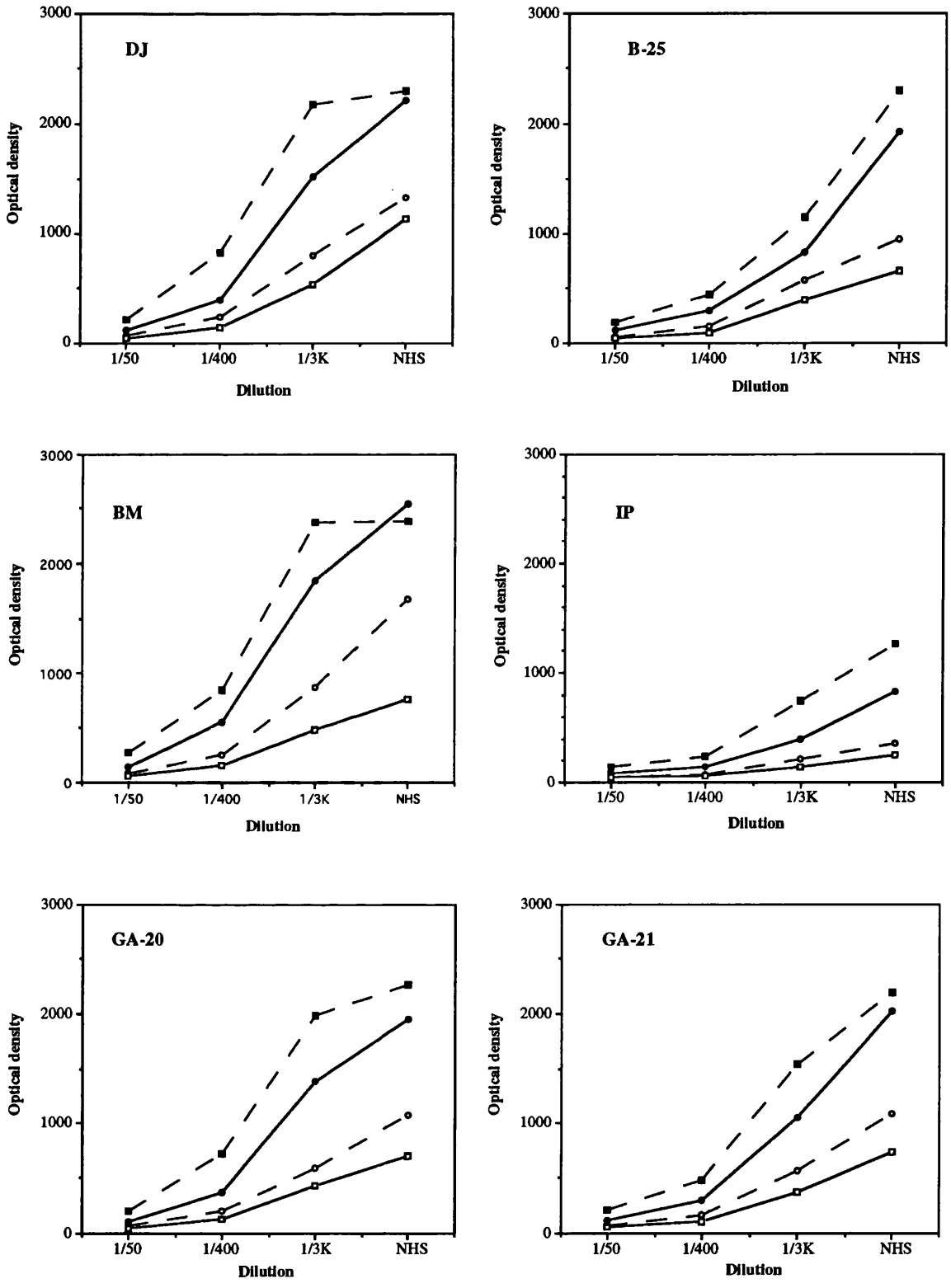


Figure 3.9. Assessment of six anti-HIV-2 conjugates. Optical densities (X 1000) of six HIV-2 conjugates plotted against serum dilution at the following conjugate concentrations:

—■— 1/250 —●— 1/500 —○— 1/1000 —□— 1/1300

from HIV-2 infected individuals would serve as effective conjugates in conjunction with the recombinant protein.

iii. Reactivity of SIVagm, SIVsm and SIVmac sera.

Ten sera obtained from monkeys infected with SIVagm were reacted undiluted in both the HIV-1 competitive EIA and the HIV-2 competitive EIA (VC-EIA). Standardised optical densities were calculated in order to compare the reactivity of these sera (Figure 3.10.). All ten sera were negative for anti-HIV-1 with two of these also unreactive for anti-HIV-2 whereas eight sera (AGM 14, AGM 20, AGM 23, AGM 25, AGM 27, AGM 30, AGM 53 and AGM 57) were all strongly reactive in the HIV-2 VC-EIA.

The extent of reactivity was investigated further since >90% inhibition for each of these was observed. This was intriguing because of the extremely high level of inhibition which was more similar to the strong reactivity obtained with natural HIV-2 infection and was in direct contrast with the complete lack of reactivity of these sera with the HIV-1 assay. The reactivity was therefore highly specific for anti-HIV-2 only. To investigate the level of reactivity with HIV-2 antigens, each serum was titrated in NHS at 1/10, 1/100, 1/1000, 1/2000, 1/4000 and 1/8000 dilutions and the degree to which conjugate inhibition occurred compared. The regression curves indicated that both low and high levels of antibody were present as shown in Figure 3.11. Indeed, the extent of reactivity of some of these, in particular AGM 14 and AGM 20 which have 50% end-points of >5000 is comparable with some of the highest titres obtained with sera from individuals naturally infected with HIV-2. The same dilutions were also reacted in the RC-EIA with similar findings, whereby reactions were of comparably lower sensitivity as previously found with HIV-2 sera (Figure 3.12.). This lower level of cross-reactivity in the recombinant assay is once more most likely due to reduced senstivity of this assay since the reduction occurred with all three serum groups. The lack of *gag* epitopes may be one explanation of this.

However, extensive cross-reactions between the antibody responses to SIVmac, SIVagm and HIV-2 was clearly demonstrated by assaying $\log_2$ dilutions of each sample. Although both SIVmac 32H and AGM 14 sera are of simian origin, it was clearly demonstrated

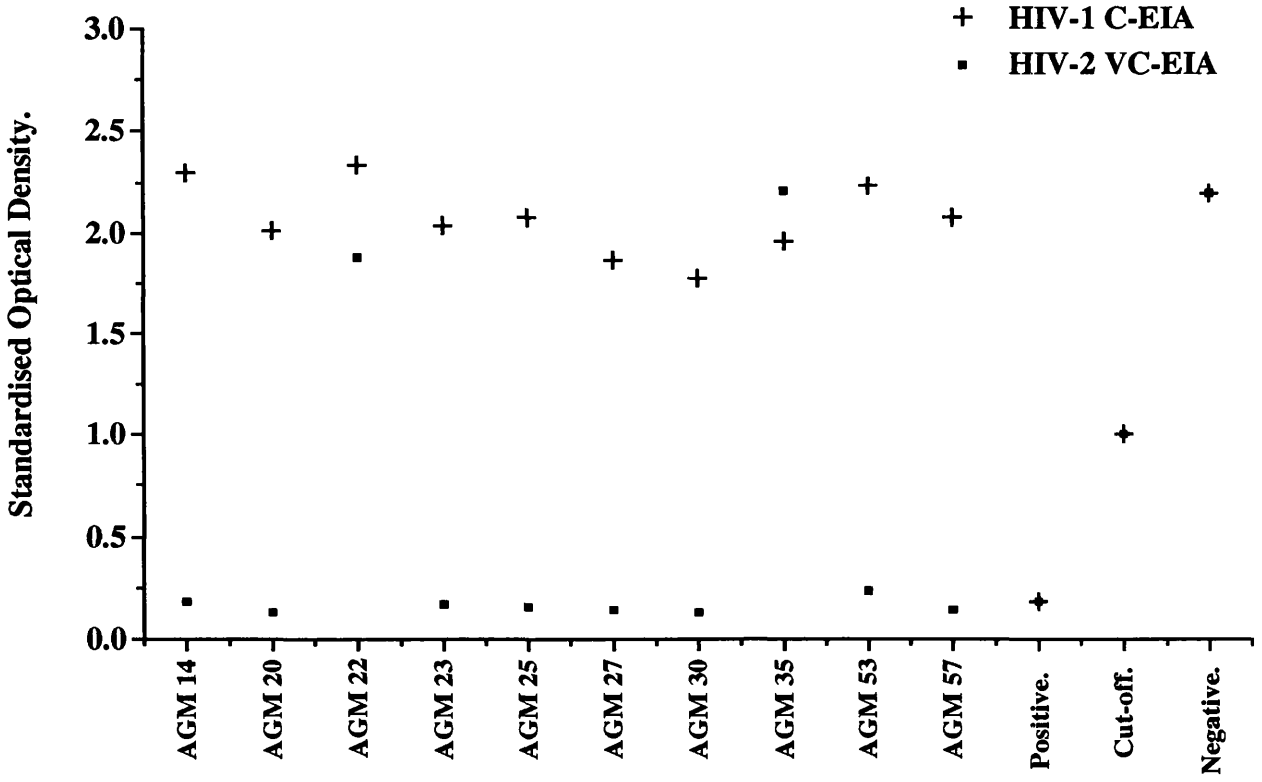


Figure 3.10. Reactivity of 10 sera obtained from African green monkeys by HIV-1 and HIV-2 competitive EIAs. The HIV-2 VC-EIA was used and standardised optical densities were calculated for each assay.

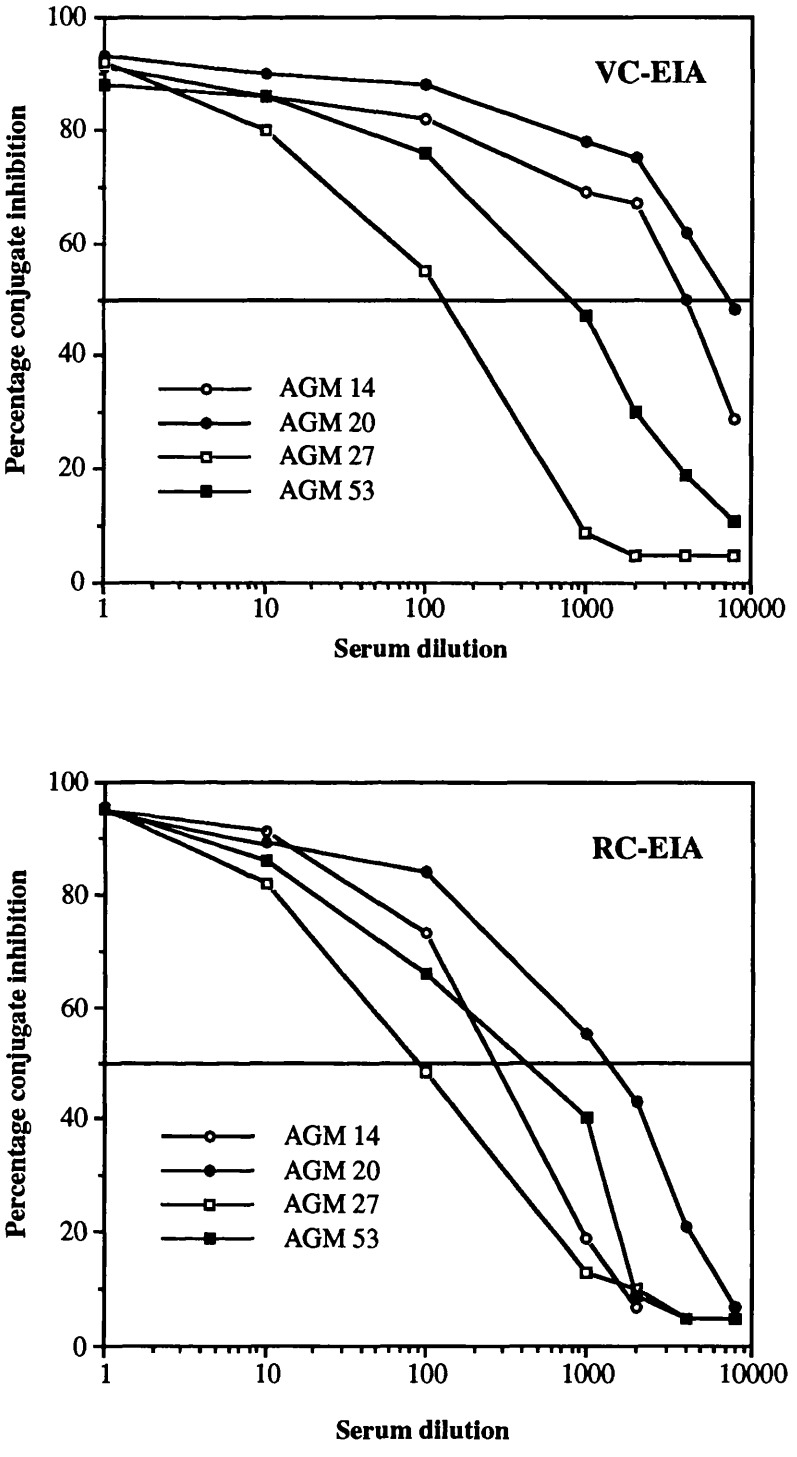


Figure 3.11. Reactivities of four AGM sera in HIV-2 competitive EIAs. Percentage conjugate inhibition of four AGM sera in the VC-EIA (upper) and the RC-EIA (lower) are shown.

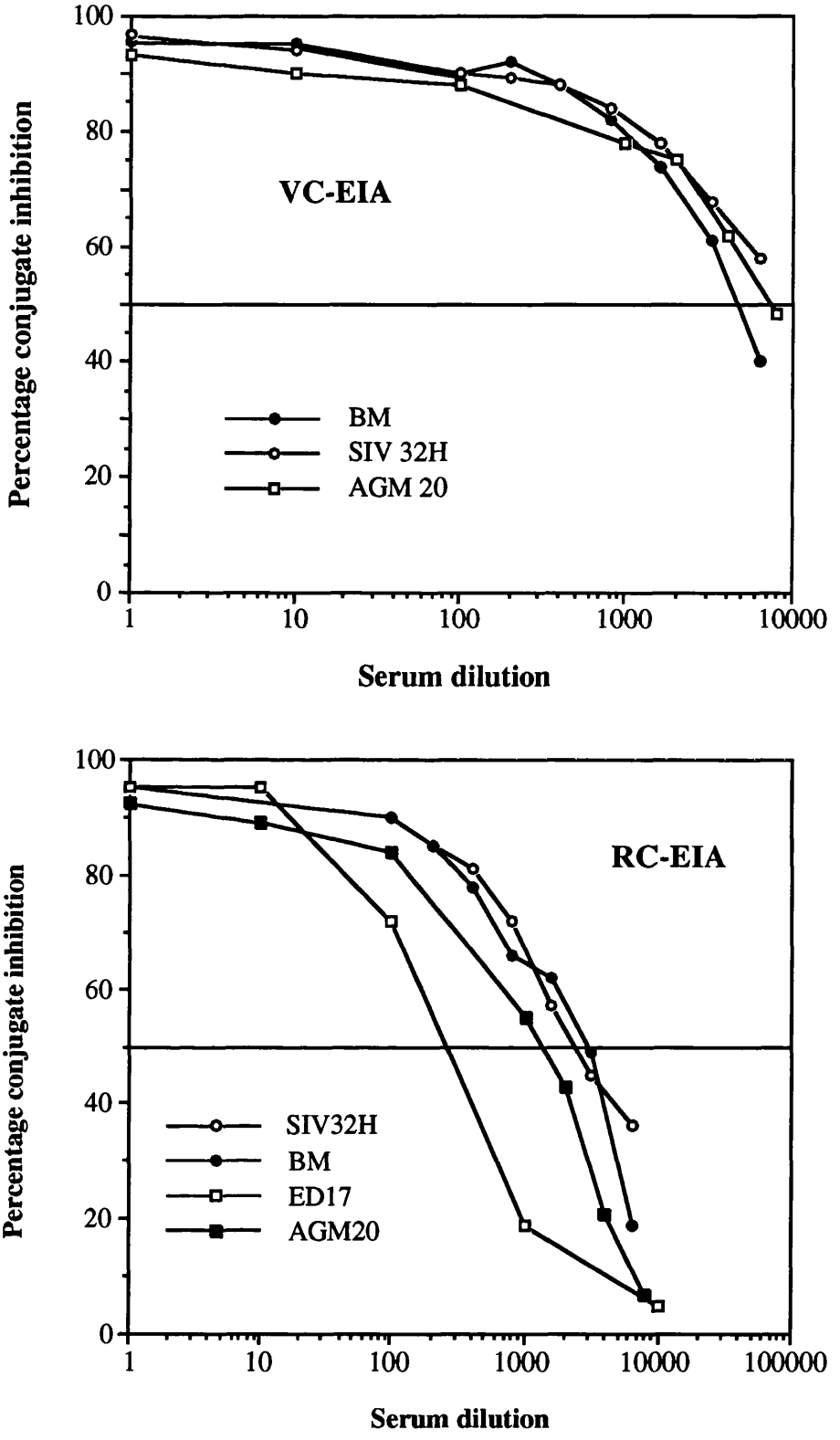


Figure 3.12. Comparison of reactivities of SIV and HIV-2 sera in viral lysate and recombinant competitive assays.

Sera tested by competitive EIA :

SIV 32H : SIV mac BM : HIV-2

ED17 : Sooty mangabey AGM 20 : African Green Monkey

that both exhibit very strong reactivity with HIV-2 *env* epitopes within the TMP which was strongly antigenic for these samples. The sooty mangabey serum sample tested in the RC-EIA assay also titrated out to significant levels, beyond that observed with weak antibody reactivity with HIV-1 sera. Although antibody levels were not as strong with this sample as the AGM samples, this may be merely a reflection of low level specific antibody in the monkey rather than its inability to bind with HIV-2 antigens.

3.c. Discussion.

3.c.1. Competitive EIAs for the detection of anti-HIV-2.

The development of competitive assays utilising reagents originating from the MRC unit in The Gambia was an important feature of establishing a programme of research into the natural history of HIV-2 and comparing the clinical importance of HIV-1 and HIV-2 infections in The Gambia. The established HIV-2 competitive EIA incorporating viral antigens derived from purified supernant fluid of HIV-2-infected cultures, which had been established previously, was evaluated both on HIV-2-positive sera tested in London and over a period of several years in field studies in Fajara.

The VC-EIA was demonstrated to be a specific assay which was found to be generally more reliable than the ELAVIA-2 assay, an alternative monotypic assay which was of antiglobulin format. This was particularly apparent when both assays were compared by screening the same sera from a rural part of The Gambia with low prevalence of infection. Improved specificity was demonstrated using competitive assays compared with an antiglobulin assay, ELAVIA-2. In this study, 1% of sera were found to be falsely positive by the competitive EIA as opposed to 6.1% when the ELAVIA-2 assay was used and weak reactions were less of a problem with sera tested by competitive EIA. However, the cost of confirmatory testing with expensive Western blots can be considerably reduced if a primary screen with the competitive assay is followed by further testing of positive sera with ELAVIA-2. Sera which react strongly in both assays can be taken to be true positives and therefore the need for time-consuming and expensive Western blots could be largely dispensed with. This strategy is gaining recognition, using a second EIA preferably of different format, as an alternative to the use of Western blots as the principal means of confirming the presence of specific antibody. Strategies and algorithms obviating the need for Western blots are under review (Tamashiro *et al*, 1993). Similar findings have been reported where synthetic peptide EIAs have been used to confirm the initial reactivity of sera from the Ivory Coast in a screening test (De Cock *et al*, 1990c). Sera which react weakly in either assay or strongly with one and weakly with the other still require additional confirmation, however, and may still require testing by Western blot. Concordant results with two

EIAs though points to a high predictive value and challenges the dogmatic use of Western blot as a viable confirmatory assay.

The preparation of the solid-phase for the VC-EIA and the balance of antigen with conjugate produced an assay which due to the competitive format was specific for anti-HIV-2 and was more sensitive than any of the other assays with which it was compared. The ability to produce enough of this relatively pure culture-derived antigen, however, has been a limiting factor in the application of competitive assays to field studies. Reproducibility experiments showed that the competitive assay, like other EIAs, is not in practice perfect and it is necessary to use two independent tests, preferably EIAs of different formats and repeat both of these on a second serum sample. However, over a period of three years the competitive assay (VC-EIA) was proven to be a specific and sensitive means to identify individuals infected with HIV-2. The assay is relatively easy to perform, being of simple format which is both robust and durable. The balance of critical reagents, however, is central to a well-optimised assay. It had been previously shown that DJ was a good conjugate with CBL-20 antigen (Tedder *et al*, 1988). The time and cost of producing a tissue-culture derived antigen, however, is a significant disadvantage and therefore replacing it with a recombinant antigen greatly facilitated the preparation of the solid-phase.

Therefore a recombinant antigen was used to replace the culture-derived antigens although it was opted, in the first instance, to maintain conjugated DJ serum as the detection reagent since a large source of conjugated and unbound plasma was available. Subsequent experiments, however, indicated that other HIV-2 sera would perform equally well, and possibly better than the DJ conjugate. The recombinant protein was insoluble with mild denaturing agents, although under strong denaturing conditions with high concentrations of SDS yielded a soluble preparation which was demonstrated to be highly antigenic. The removal of these impurities and unbound bacterial proteins was mediated by washing the solid-phase and since the β-galactosidase component of the crude antigen preparation remains bound to the capture antibody, this process acted as an efficient affinity-purification step.

High levels of virus expression were required to produce a sensitive assay with the VC-EIA direct coat, utilising CBL-20 as the source of antigen. Prolonged and continual expression of CBL-20 to high titres became difficult to achieve over many months and was a limiting constraint in producing this assay. If a weak antigen preparation is used, the reactivity with the conjugate may be enhanced by increasing the amount of conjugated antibody available for binding. The result of this, however, is to reduce the sensitivity of the assay since this is dependent on the ability of specific antibody to compete with the conjugate. Sensitivity was determined using a panel of HIV-2 sera which were each tested in both competitive assays (VC and RC-EIA), the ELAVIA-2 assay and each of the combined assays. The level of cross-reactivity between HIV-1 and HIV-2 is by no means complete and the HIV-2-specific component, particularly in combination assays is therefore essential for efficient detection of anti-HIV-2. However, the direct incorporation of an HIV-2 component into a combination assay which is designed to detect optimally anti-HIV-1 may compromise both specificity and sensitivity and new assays designed on an empirical basis for the simultaneous detection of anti-HIV-1 and anti-HIV-2 are likely to perform better than others where an HIV-2 component has merely been added into the assay format. The choice of antigen as well as assay format is also important.

Synthetic peptides representing the N-terminal region of the transmembrane glycoprotein of HIV-2-related viruses were initially used in site-directed serological assays to screen for anti-HIV-2 (Norrby *et al*, 1987) and applied in an epidemiological survey of HIV-2 infection in Guinea Bissau, West Africa (Norrby *et al*, 1989). A single epitope, the WGCAFR motif within gp36, was found to represent the major immunodominant region and defined much of the specific anti-HIV-2 reactivity. This assay failed to detect only a single anti-HIV-2 positive serum among 90. In the present study where gp36 peptides have been used and geometric mean titres (GMT) determined for 15 sera, the assays performed generally well in their ability to detect HIV-2-specific antibody (Enzygnost GMT = 613; Wellcome 1+2 GMT = 1448). This was better than either a cell-culture-derived antigen or a recombinant antigen (ELAVIA Rapid Mixt = 386, Abbott = 232 respectively). This may, however, simply be a reflection of the kinetics of employing a mixed antigen preparation on the solid-phase where the HIV-2 component has been

incorporated into an existing HIV-1 assay. None of the commercial assays, including ELAVIA 2 (GMT = 1273), however, were able to match the sensitivity of the competitive EIA for anti-HIV-2 when viral lysate antigens were used (GMT = 2670) which was demonstrated to be a sensitive means of detecting HIV-2 antibodies.

3.c.2. Specificity of HIV-1 and HIV-2 serological assays.

It is still unclear, however, whether assays can be made to detect only a type-specific response. Some sera are capable of exhibiting strong cross-reactivity with the heterologous virus. It remains difficult to design an assay which exhibits no reaction with the heterologous virus and yet still remain sensitive enough to detect reliably all sera containing anti-HIV-2 (Bottiger *et al*, 1990). Recombinant proteins have been employed for the specific detection of anti-HIV-2 (Zuber *et al*, 1990; Schulz *et al*, 1989 and 1988) However, the ability to detect solely the anti-HIV-2 response, in the presence of cross-reactive HIV-1 antibodies, remains difficult to achieve. The conservation of gp36 appears to be high although variation in the antigenic region of HIV-1 gp41 in some African strains have also been described which may affect antibody recognition (Lange *et al*, 1993).

Although competitive assays represent a more specific assay format than other assays for detection of anti-HIV, cross-reactions with the heterologous antigens are nevertheless demonstrable with competitive assays. Linear B-cell epitopes within *gag* have been identified for both HIV-1 and HIV-2 (Janvier *et al*, 1990). Cross-reactions were observed both with the viral lysate antigen which contains both *gag* and *env* components, and the recombinant antigen which relies purely on anti-*env*-specific reactivity. However it was possible to demonstrate reduced reactivity in sera containing anti-HIV-1 with the anti-HIV-2 recombinant assay compared with the original assay based on the viral lysate antigen which is likely therefore to be a reflection, in part, of the absense of cross-reactive *gag* epitopes of this antigen. Clearly, some cross-reactive epitopes within the region of the transmembrane glycoprotein expressed exist and it is likely that these are also responsible for some sera exhibiting a strong response to both the homologous and heterologous virus. This becomes particularly apparent with the sera which gave greater than 90% inhibition when tested undiluted in all three competitive assays.

In a study in the Ivory Coast, cross-reactivity of antibodies to heterologous antigens have been investigated using Western blots and synthetic peptide tests on sera from tuberculosis patients and blood donors (DeCock *et al,* 1991). Dual serological profiles appear common in this region of West Africa possibly reflecting a high proportion of both virus-types co-circulating, although serological reactivity to both viruses by no means indicates dual infection. This type of ambiguous dual reactivity when observed in sera from The Gambia was resolved in most cases at dilutions of 1/10 and 1/100 which then become relatively easy to assign as either HIV-1 or HIV-2 predominant.

Sera which exhibited identical regression lines with relatively high levels of antibody to both viruses in all three assays and with antibody titres exceeding 1,000, or in some cases over 10,000 in each assay, are most suggestive of dual infection or possibly infection with a HIV-1/HIV-2 recombinant. Some sera, both those described here and in other studies (Bottiger *et al,* 1990; Simon *et al,* 1989; Rayfield *et al,* 1988; Rey *et al,* 1987), are capable of exhibiting a strong response to both viruses. However, only those sera which have equivalent reactivity by whole virus EIAs, Western blots, RIPA and against specific peptides are most likely to be indicative of a dual infection or a recombination variant. It has yet to be demonstrated, either *in vivo* or by *in vitro* genotype recombination experiments that variants can arise containing mixtures of the two genomes which might account for the strong cross-reactions observed at the antigenic level. Indeed, if such viruses do occur it seems more likely that this new hybrid would react more weakly with HIV-1 or HIV-2 antigens, in particular the *env*-antigens, rather than give an equally strong response to both. The detection of HIV proviral DNA by amplification techniques was applied to some of these samples as described in Chapter 5.

Dual infections are likely to become more common as HIV-1 spreads in West Africa and speciation of HIV infections on the basis of serological criteria remains of high priority. In the absense of a competitive EIA for anti-HIV-2, it has been possible to identify sera likely to contain HIV-2 antibody on the basis of their reactivity in the HIV-1 competitive assay (Simon *et al,* 1990). Re-evaluation of indeterminate HIV-1 sera may in some cases also be used to identify HIV-2 reactivity with other assay formats (Myers *et al,* 1992).

With the availability of combined HIV-1 and HIV-2 assays which can screen efficiently for both viruses simultaneously, independent monospecific competitive assays can be used effectively to confirm the anti-HIV specificity of sera positive in the primary screen. The strategy of using combined assays in the primary screen followed by HIV-1 and HIV-2 competitive assays and another assay may provide an effective algorithm which could be used to supplant Western blots as confirmation assays. A further anomaly of testing for HIV-2 antibodies by Western blots is the transmembrane existing as oligomers as gp80 (Rey *et al*, 1990). Anti-transmembrane antibodies may react with the gp80 moiety rather than with the monomeric gp36/gp41 form. A smear or 'blush' of antibody reactivity with the monomeric form of the TMP is often considered to be diagnostic for anti-transmembrane reactivity, rather than discrete bands occuring at the gp36/gp41 position. Reactions with gp80 could therefore be mis-leading. It has been proposed that disassociation of the oligomeric forms can be achieved with trichloracetic acid resulting in monomeric gp36/41 and can circumvent this phenomenon (Parekh *et al*, 1991). Taking the general problems associated with the use of HIV-2 Western blot testing into account, a strong case can be made for alternative strategies for confirming the specific reactivity of test sera.

With this in mind, a competitive EIA for anti-HIV-2 may gain in use, particularly as HIV-2 increases in prevalence in countries where HIV-1 infection is already established. A change in WHO testing strategies has been discussed (Tamashiro *et al*, 1993) and a competitive HIV-2 assay would be a strong candidate for use in such situations. Competitive assays have a proven record both in terms of specificity and sensitivity in a field situation as front-line screening assays (VC-EIA) and when applied to sera exhibiting ambiguous dually reactive profiles (VC-and RC-EIAs). The use of a site-directed serological competitive assay (RC-EIA) therefore has an important role to play in confirming the specificity of sera reacting in a primary combined screen.

3.c.3. Detection of related lentiviruses.

Site-directed serological assays also represent important and useful tools not only in seroepidemiological studies but also in examining the relationship between related simian lentiviruses. These have been used to potentially distinguish between HIV-1, HIV-2 and

SIV lentivirus infections by targetting the C-terminus of the external glycoprotein where an epitope located within the last 13-15 residues may be related to the geographical origin of the sera (Baillou *et al*, 1991). This part of the EGP, however, is not recognised by all positive sera although in some cases the gp120 C-terminal epitope may be useful in distinguishing the HIV-2/SIV serotype.

The antigenic relationship of some non-human primate lentiviruses with HIV-2 was investigated in this thesis by analysing sera from SIVmac, SIVsm and SIVagm-infected monkeys. Reactivity with all three groups of sera was demonstrated by HIV-2 competitive EIA which in the case of SIVmac and SIVsm sera would perhaps be expected since they are members of the same phylogenetic group. The reactivity of the SIVmac serum sample is similar to that observed by Barin *et al,* (1985) before HIV-2 had been isolated although more serum samples from SIV-infected sooty mangabeys and macaques would be required to confirm the ability of the HIV-2 competitive EIAs to detect a spectrum of related simian lentiviruses. The strong reactivity of the AGM sera, however was unexpected and surprising, particularly in view of the lack of reactivity with the HIV-1 competitive EIA. Indeed, titration of antibody levels to end-point values indicated that four of the AGM sera could inhibit the conjugate binding with high efficiency, to the extent with which this would occur by antibodies derived from natural infection with HIV-2.

One of the applications of competitive assays and apparent advantage over anti-species immunoassays, therefore, is for screening of related lentiviruses in feral populations of monkeys. Provided epitope recognition occurs between test antibody and the antigen used in the assay, inhibition of the conjugate will occur irrespective of the species under investigation. The SIVagm sera also reacted strongly with the recombinant HIV-2 antigen which, in comparison to the culture-derived preparation, does not contain a *gag* component resulting in only a slight loss in sensitivity. Although no direct link with SIVagm and human lentiviral infection has been identified, evidence for transmission between SIVsm infection in sooty mangabeys and human HIV-2 infection has been proposed (Gao *et al*, 1992). The possible role and significance of simian lentivirus infection in human disease remains to be fully evaluated and it is possible other

undetected, related immunodeficiency viruses in feral monkey populations may exist. The detection of antibodies by competitive assays may be useful in investigating hitherto unknown lentiviral infection.

In summary, the use of competitive EIAs for the detection of anti-HIV-2 and the continued supply of these reagents to collaborators in West Africa have played an important role in establishing studies into HIV-2 infection. The data presented for the VC-EIA indicated this assay to be both sensitive and specific, and the suitability of the RC-EIA as a confirmatory assay in The Gambia is currently in progress. The simple assay formats make them readily amenable to screening large numbers of sera, particularly in blood transfusion centres. The HIV-2 assay has also been used at the Royal Victoria Hospital, Banjul, in this context as well as at the MRC laboratories in Fajara. With the advent of combined HIV-1 and HIV-2 screening, the role of these assays has changed with the need to confirm and speciate the reactivity of the infecting agent has become the major application of these tests. Competitive assays, in conjunction with other anti-HIV-1 and HIV-2-specific assays could play an important role in supplanting the Western blot as the mandatory means of confirmatory testing. At the current time, however, this remains a matter of conjecture although with an increasing global presence of HIV-2 this matter is likely to be placed higher on the agenda of organisations recommending testing algorithms for HIV testing.

Chapter 4.

HIV-2 *gag* p26 studies.

4.a. Introduction

The *gag* proteins of HIV constitute the core of the virion, and play an important role in the processes of virus assembly and budding. In HIV-1 and HIV-2, the precursor polyprotein, p55 is post-translationally modified to yield a matrix protein (p16) which surrounds the major virion protein of HIV-1 (p24) or HIV-2 (p26). Within this is the nucleocapsid protein, p12. Post-translational modification occurs via HIV-specific proteases and the amino terminus is blocked by the addition of myristic acid. Non-myristyolated *gag* proteins are not present in intact, processed virions and myristyolation directs the *gag* precursor to the inner surface of the cytoplasmic membrane. The *gag* proteins therefore play a central role in virus assembly since retroviral RNA also interacts with the nucleocapsid protein (p9) region of the *gag* precursor before packaging (Wills and Craven, 1991, Gelderblom, 1991).

The detection of HIV-1 core antigens in the serum of HIV-1-infected individuals has frequently been used as an indication of virus load and the measurement of p24 antigen (p24Ag) has served as a prognostic marker for HIV-1 disease, since symptomatic patients are more likely to be p24 antigenaemic than asymptomatic ones (Allain *et al*, 1986; Lange *et al*, 1989). Furthermore, the quantification of p24Ag can be used as a measure of virus expression in HIV-infected cultures, for determining the biological and growth characteristics of established or novel isolates. Classical methods used to determine the presence of a retrovirus in permissive cell lines have relied upon the detection of reverse transcriptase enzymatic activity, though these techniques require additional expertise, are not routinely performed by most laboratories and assays for detection of p24 antigens have provided a convenient substitute.

Antigen assays for HIV-2 were developed, including the production of a recombinant HIV-2 *gag* p26 antigen. The pGEX expression vector system was used to clone and express PCR-amplified p26 from the CBL-20 isolate to produce a glutathione S-transferase (GST) fusion protein using the vectors originally described by Smith and Johnson, (1988). The DNA of the four HIV-2 CBL isolates were also sequenced in parts

of p26 and their phylogenetic relationship to each other and HIV-2_{ROD} determined. The antigen assays developed were used to determine the relative expression of HIV-2 in culture fluids and their suitability for measurement of virus load in HIV-2-infected patients is discussed.

4.b. Results.

4.b.1. Detection of HIV-2 antigens.

i. HIV-2 antigen cature assay.

Solid-phase was prepared from a serum obtained from an HIV-2-infected Gambian individual (T1623) which had been shown to contain high levels of HIV-2 antibody by competitive EIA and reacted strongly with all the major structural proteins by Western blot. The serum was partially purified by precipitation with ammonium sulphate and coated at a dilution of 1/1000 (approx 5-10μg/ml as determined by spectrophotometry). The detecting antibody used in serological studies (DJ conjugated to HRPO) signalled in preliminary experiments with purified supernatant fluid obtained from HIV-2-infected cultures using TMB as the substrate. However, to increase sensitivity of this assay the DJ serum was conjugated to alkaline phosphatase (DJ-AP) and used in conjunction with an amplification reaction (Figure 2.7.). Inactivated supernatant fluid from HIV-2-infected cultures (CBL-20, CBL-22 and LAV-2_{ROD}) were used to assess the ability of this assay to detect HIV-2 antigens. Supernatant from uninfected cultures were used as controls.

Titrations of the DJ-AP conjugate were made and tested against aliquots of 'bulk' supernatant from CBL-22 and LAV-2 which were tested in duplicate with the conjugate at dilutions of 1/500, 1/1000, 1/1500 and 1/2000. Supernatant from C8166 uninfected cultures provided a measurement of background reactivity. The results of these experiments indicated that a conjugate dilution of 1/1000 was optimal providing optical densities of >2000 (OD X 1000) with backgrounds obtained with negative supernatants having optical densities <200 (OD X 1000). This assay was used to test the time-course series of samples.

ii. Comparison of antigen assays with reverse transcriptase activity.

A time-course experiment was established as described in section 2.c.3.ii. For each virus (CBL-20, CBL-22 and LAV-2_{ROD}), the relative levels of HIV-2 expression were also determined by measuring the level of reverse transciptase enzymatic activity. Antigen levels were measured using the DJ-AP amplification assay. All three HIV-2 strains exhibited broadly similar growth patterns although significantly different levels of virus were shed into the supernatant indicated by varying reverse transcriptase levels. Counts

per minute <1000 were taken as background. Antigen levels and reverse transcriptase activity were compared for each virus (Figure 4.1.). Some residual virus was carried over from the primary cultures in order to establish the time-course, which was reflected in a small pulse of RT activity before the exponential phase of growth. However, comparison of the three HIV-2 time-course cultures indicated some interesting trends.

The prototypic strain, LAV-2_{ROD}, was included in these experiments since it has been well adapted to culture and compared with CBL-20 and CBL-22. Of the three isolates, HIV-2_{ROD} produced the highest amount of virus, both in terms of RT activity and reactivity by EIA. The accumulation of viral antigens was maximal by 6-8 days and was immediately preceded by a drop in RT activity. Comparison of the CBL-20 and CBL-22 growth curves and antigen level profiles indicated that CBL-22 induces the release of virus more quickly into the supernatant than CBL-20 which was associated with a lower level of expression and a gradual accumulation of viral antigens in the supernatant. This supports earlier experiences of culturing CBL-20 and CBL-22 since CBL-22 was found to produce virus more efficiently than CBL-20. The specificity of this assay was also good, with no cross-reactivity with CBL-1 antigens detectable, in spite of there being moderately high levels of p24 antigen available for cross-reactions to occur. These comparative studies served as a useful guide for establishing an HIV-2 antigen capture assay using basic reagents. Differences in the growth properties of CBL-20 and CBL-22 were demonstrated, as well as a complete lack of reactivity with HIV-1 antigens.

Antigen levels were also independently assessed using the Innotest assay for all four isolates and the response to each of these in this assay is shown in Figure 4.2. The HIV-2 supernatants signalled only very weakly in the Innotest assay, even when viral production was at high levels as demonstrated by the HIV-2-specific antigen assay and RT levels. This assay, however, was reasonably proficient at HIV-1 antigen detection with antigen levels ranging from 64-300pg/ml using the control samples provided with the test. On the basis of these data, this assay would be unsuitable for HIV-2 antigen detection in HIV-2 studies although would probably be adequate for similar studies of HIV-1 antigen detection.

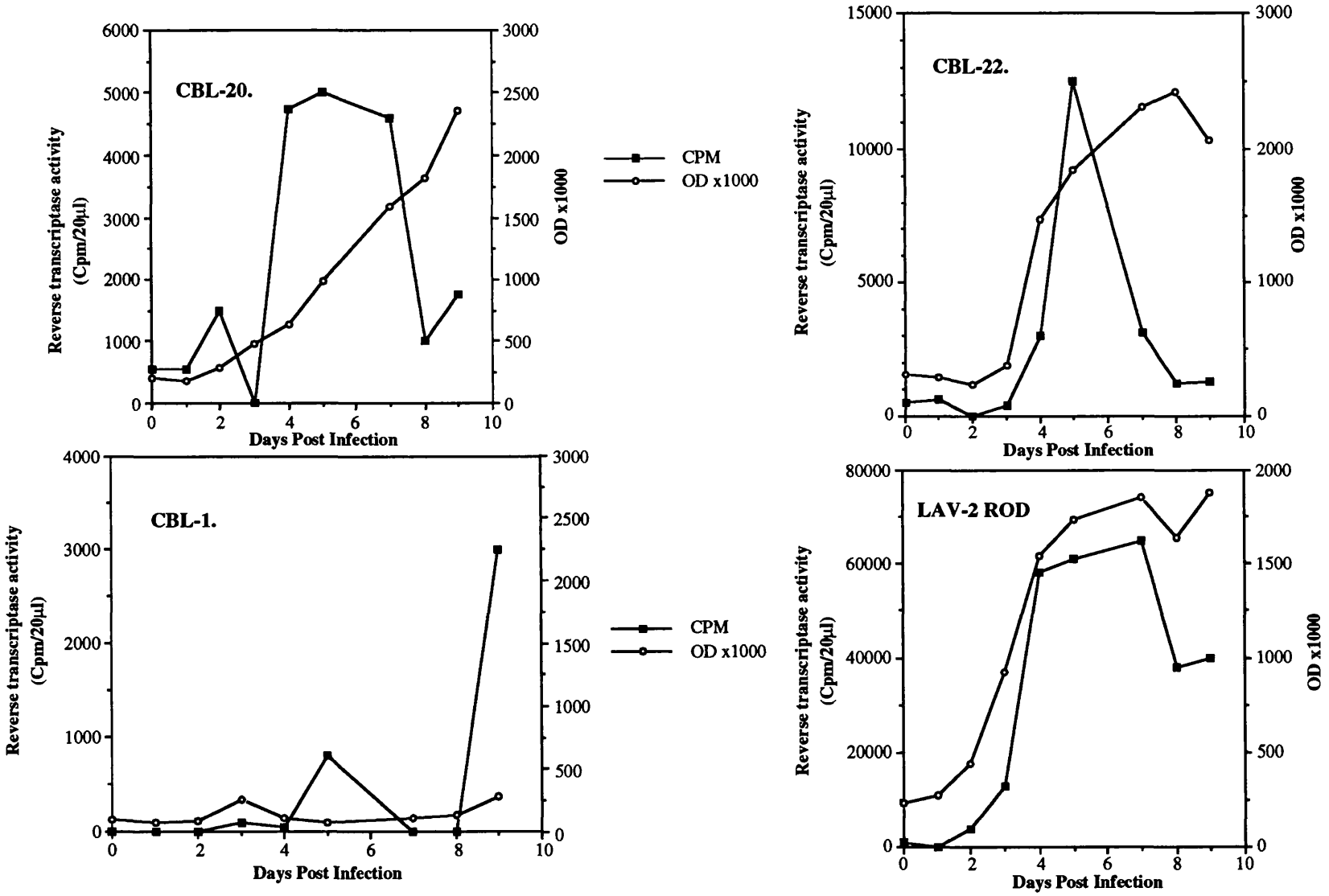


Figure 4.1. Comparison of reverse transcriptase activity and antigen production in time-course experiments.

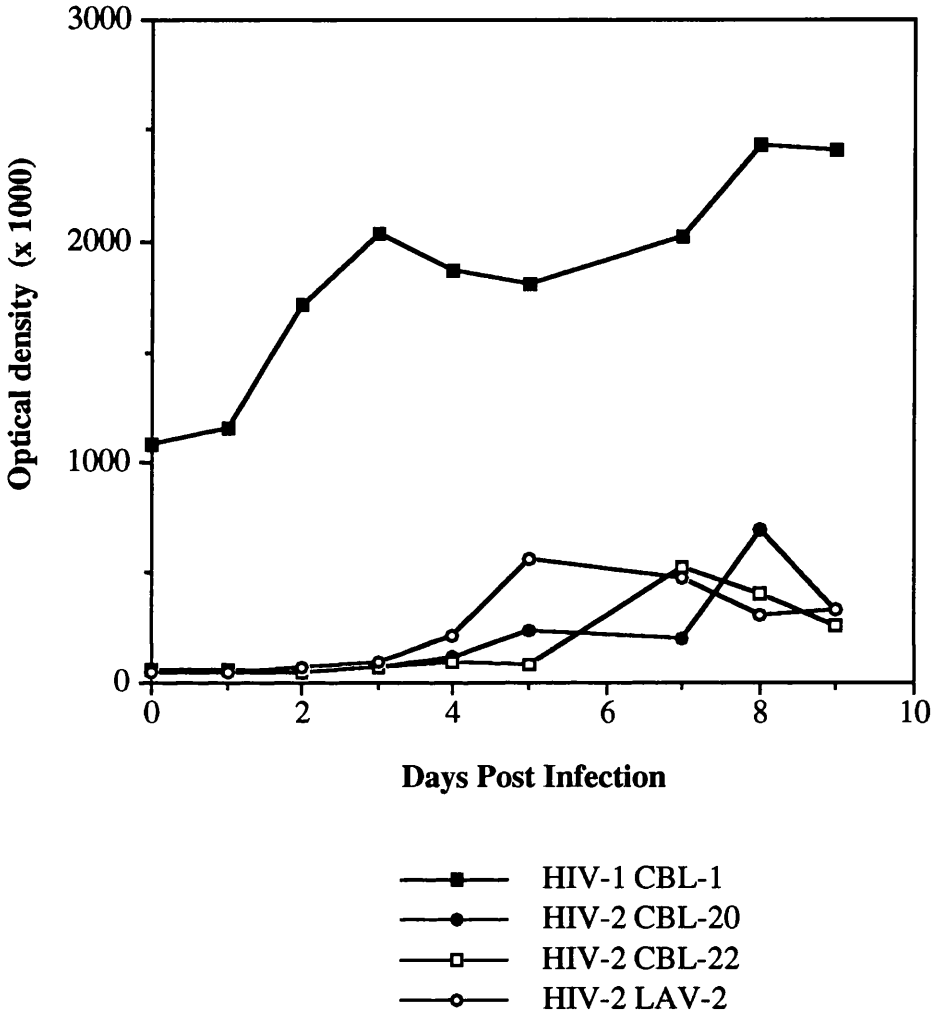


Figure 4.2. Comparison of HIV-1 and HIV-2 infected antigen levels using a commercially available antigen assay. Supernatant fluids were assayed from HIV-1 and HIV-2-infected cultures in the Innotest (Innogenetics Ltd) antigen assay.

4.b.2. HIV-2 GST-p26 recombinant fusion protein.

Recombinant HIV-2 p26 was produced by expression in bacteria using the pGEX-3X expression system as described in Chapter 2. The p26 gene of HIV-2 CBL-20 was cloned into the pGEX-3X expression vector and expressed as a GST-p26 fusion protein. Subsequently, a polyvalent antiserum was raised in rabbits using the recombinant p26 protein as an immunogen.

i. Purification of recombinant p26 by affinity capture.

The production of GST-p26 fusion proteins were tested in cultures containing the pGEX-3X/p26 plasmids. Bacterial cell lysates from both induced and uninduced cultures were analysed by SDS-PAGE. The protocols used for analysis of these proteins were followed as described in Chapter 2. A Coomassie stained SDS-PAGE gel is shown in Figure 4.3. exhibiting relatively high levels of recombinant GST-p26, compared to other bacterial proteins. The GST-p26 fusion protein (p52) is clearly visible in the induced culture (Lane 1) compared with the uninduced culture (Lane 2). Following sonication of bacterial cultures and purification by passage down a glutathione-agarose affinity column, contaminating bacterial proteins were effectively removed to give highly purified recombinant HIV-2 GST-p26 (Lane 3).

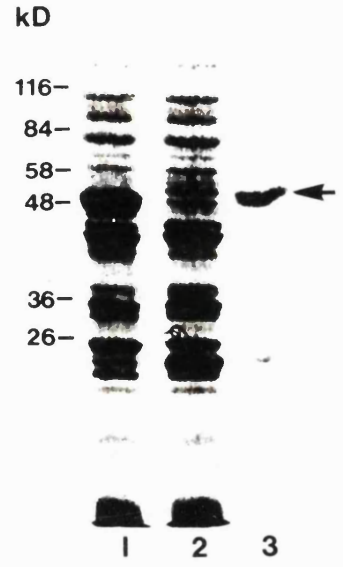
Western blots were performed using a pool of human sera containing anti-HIV-2 which reacted strongly with the purified GST-p26 fusion protein (Lane 1). Cleavage with Factor Xa yielded a doublet (Lane 2) and p26 was separated from the GST protein by a second passage down the glutathione-agarose affinity column (Figure 4.3). The purified p26 was soluble and stored at -70°C until required.

ii. Reactivity of rabbit anti-p26 by HIV-2 Western blot.

The recombinant p26 protein provided a suitable immunogen to raise a polyvalent antiserum in rabbits as described in Chapter 2. Seven days following the final injection the rabbit was bled and the anti-p26 reactivity assessed by Western blot. Figure 4.4. shows reactivity of the serum diluted 1/40, 1/400, 1/4K and 1/40K in PBS. Incubation with a goat anti-rabbit IgG conjugated to peroxidase demonstrated strong reactivity with HIV-2 p26. No reactivity was detectable at the 1/40K dilution. A strong band was visible

A) SDS-PAGE

Bacterial cell lysates from an induced (Lane 1) and an uninduced (Lane 2) *E.Coli* culture. Sonicated lysate following passage down a glutathione-agarose affinity column (Lane 3).



B) WESTERN BLOT

A pool of human anti-HIV-2 serum was used. Uncleaved GST-p26 (Lane 1) after first passage down column. Purified GST-p26 cleaved with Factor Xa (Lane 2). Purified p26 after removal of GST by glutathione-agarose chromatography (Lane 3).

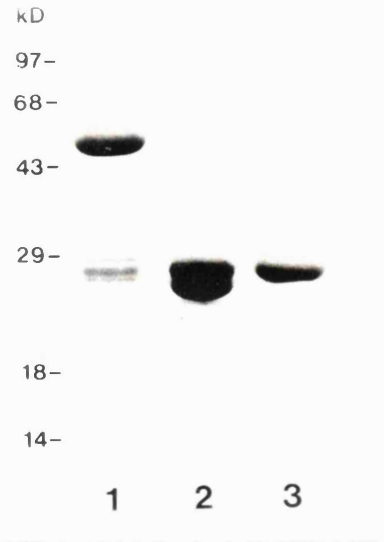


Figure 4.3. Analysis of expressed GST-p26 fusion proteins by SDS-PAGE and Western blot. SDS PAGE gels were visualised by Coomassie Brilliant Blue staining. Western blots were visualised with a pool of human anti-HIV-2 sera and then with an anti-human IgG alkaline phosphatase conjugate.

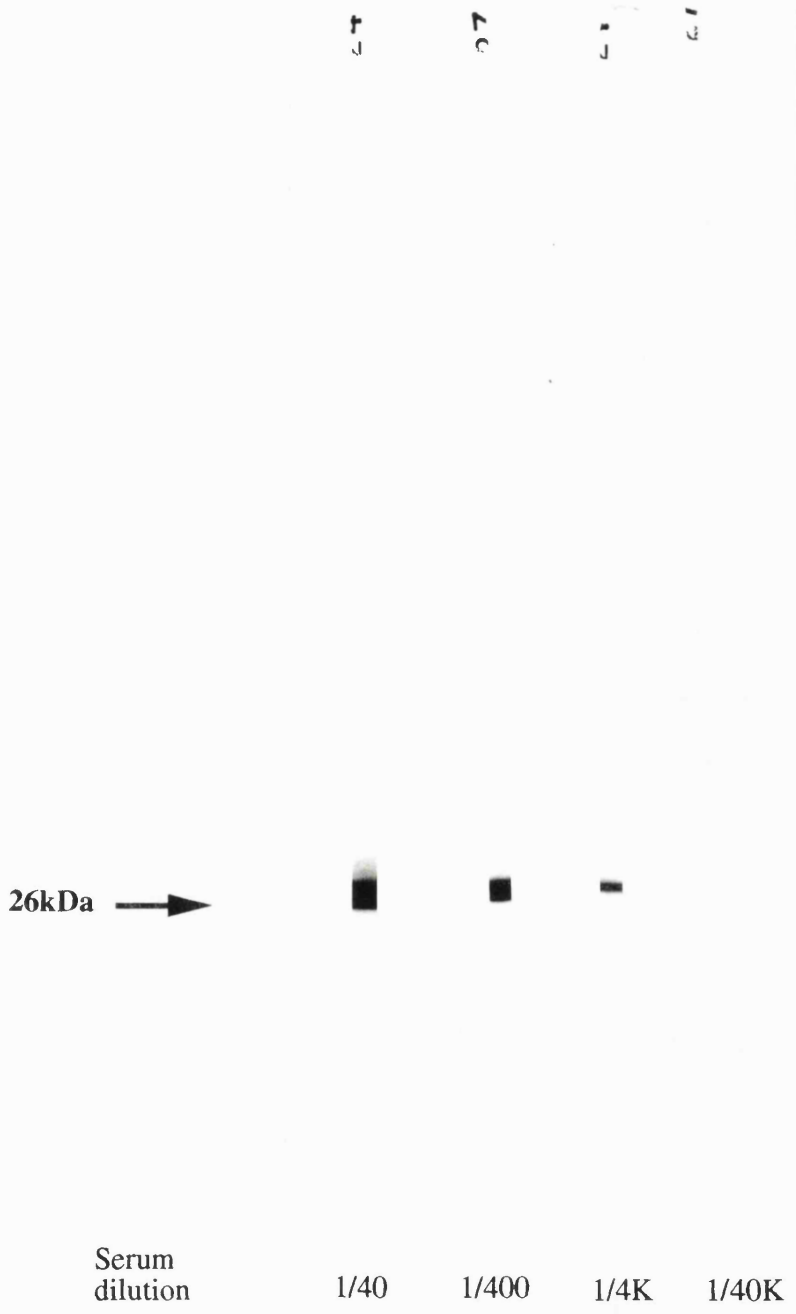


Figure 4.4. Reactivity of polyclonal rabbit anti-*gag* p26 serum on an HIV-2 Western blot. Bands were visualised with a goat anti-rabbit antibody conjugated to horse-radish peroxidase.

up to the 1/4K dilution, indicating a relatively high titre for this antiserum. This also appeared to be exclusively p26 reactivity, since no reaction was detectable with the p55 protein (Figure 4.4). The rabbit was bled completely at 10 days following the final inoculation, 20-30mls of antiserum collected and stored at -20°C.

iii. Nucleotide and predicted amino acid sequence of Gambian HIV-2 strains.

The DNA sequence of part of the p26 gene of four of the Gambian HIV-2 strains CBL.20-23 was determined using a combination of sequencing primers (Figure 2.5.). The p26 fragment was an internal nested product with the external primers amplifying the entire p55 region, the polyprotein cleavage sites which were conserved for HIV-2 isolates and SIVmac. HIV-2 p26 was sequenced with both 3' and 5' biotinylated primers in conjunction with the inverse PCR primer, or with the internal sequencing primers. Not all of the p26 gene was successfully sequenced using this approach, with the central part of this region proving difficult to sequence reliably. However, enough sequence was generated in order to assess the genetic relatedness between the four CBL isolates (CBL.20-23) and their sequence compared to that of HIV-2_{ROD} for p26 *gag*. The nucleotide sequence of these four isolates numbered according to HIV-2_{ROD} from nucleotides 966 to 1178 is shown in Table 3, Appendix 2 and from nucleotides 1512 to 1661 in Table 4, Appendix 2.

Sequences were aligned in frame with CBL-20 and CBL-22 placed together and CBL-21 and CBL-23 placed together. The number of silent and non-silent substitutions was determined and the ratio of these for the Gambian strains was 6.9 to 1 using HIV-2_{ROD} as a reference, which is similar to the degree of variability known to exist in the analogous region of HIV-1 *gag*. The sequence overall is generally highly conserved, though using the maximum likelihood method of phylogenetic analysis, CBL-20 and CBl-22 were found to group together separate from CBL-21 and CBL-23 which formed a second group, HIV-2_{ROD} forming a third branch as shown in Figure 4.5. However, translation of a larger section of sequence for CBL-20 only into the predicted amino acid sequence indicated a relatively high level of protein similarity with few amino acids differing from HIV-2_{ROD}. The CBL-20 p26 amino acid sequence was found to be representative of most of the HIV-2 strains in the database, which belong to subtype A,

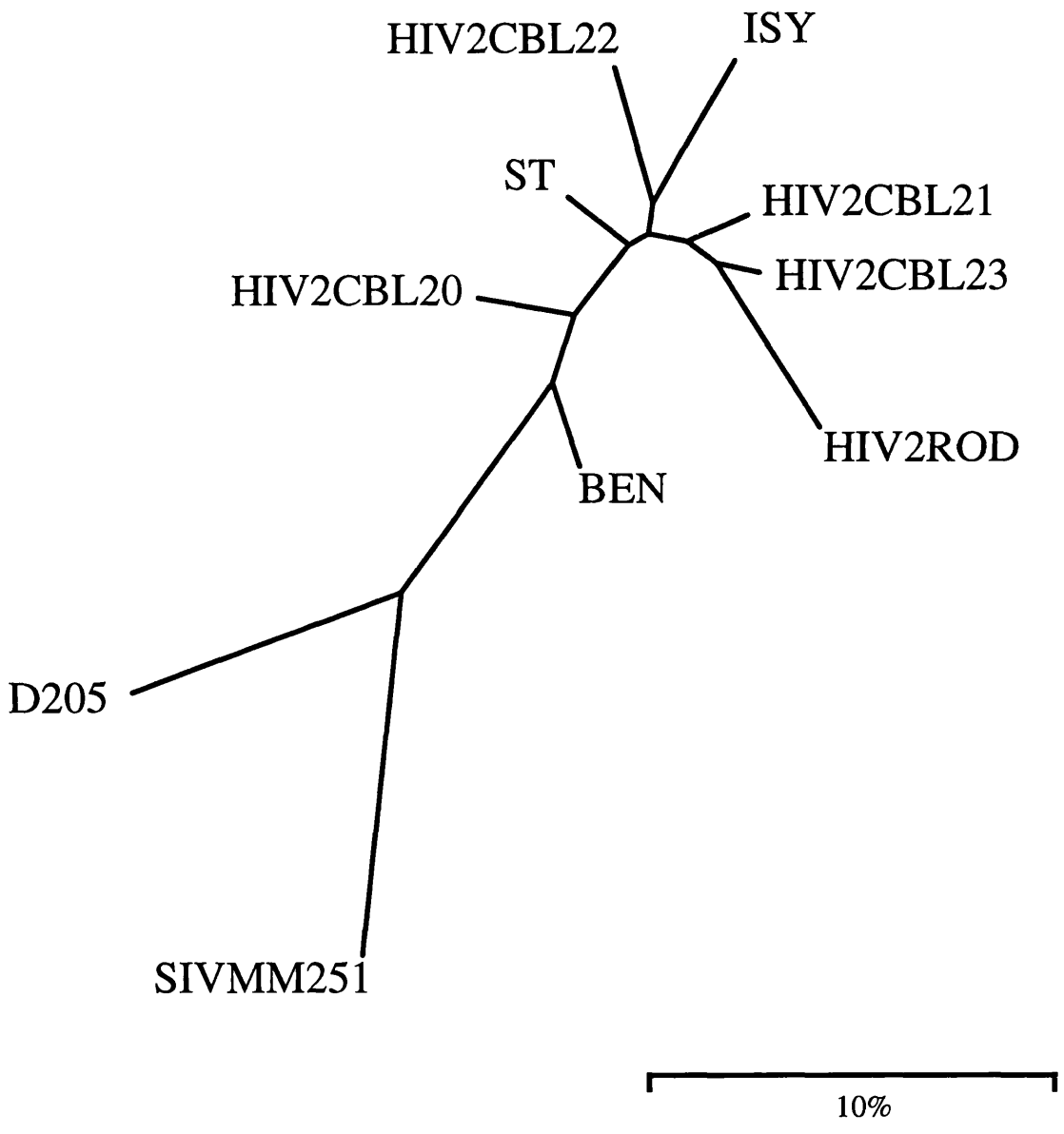


Figure 4.5 Maximum Likelihood phylogenetic analysis of HIV-2/SIV p26 gag.

Sequences were compared for CBL20 - 23 with HIV-2 ROD, ST, ISY and BEN (subtype A), HIV-2 D205 (subtype B) and SIVmm251.

and is shown in Figure 4.6. The small stretch of amino acids for which no sequence was obtained for CBL-20 appears to be highly conserved with few changes in other isolates.

These overall findings agree with the earlier findings of Schulz *et al*, (1990) showing the CBL series of HIV-2 isolates not to be too dissimilar to most other laboratory isolates of HIV-2, at least in terms of their overall sequence. Although PCR amplification, cloning and expression of HIV-2_{ROD} would probably have been sufficient for these studies, the CBL-20 strain was chosen because at the time the extent of genome variability within these Gambian strains was less clear. These studies have generated more information with respect to this material and were instructive in determining how representative the expressed protein was of a limited number of strains isolated from Gambian HIV-2-infected individuals.

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GGNYTHIPLSPRTLNAWVKLVEEKKFGAEVVPGFQALSEGCTPYDINQMLNCVGDHQAAMQIIREIINEEAAEWDVQHPIPGPLPA   ROD
------V-----------------------------------E---------------------------D--A-D--------   CBL-20
----V-L-------------------------------------------------------------D-------------   ISY
A-----V-------------------------------------------------------------D--A----------   ST
----V-V-------------------------------------------------------------D--S----------   BEN

GQLREPRGSDIAGTTSTVEEQIQWMFRPQNPVPVGNIYRRWIQIGLQKCVRMYNPTNILDIKQGPKEPFQSYVDRFYKSLRAQTDP   ROD
----D-----M--------------Y-Q-----.....---V--V-----NQ------------------   CBL-20
----D--------------------Y-QE-------------------------------S-----------------A   ISY
---------------------------------S-----------------------------------------   ST
----D--------------------Y-------------------------K---------------------------   BEN

AVKNWMTQTLLVQNANPDCKLVLKGLGMNPTLEEMLTACQVGGPGQKA   ROD
-------------------------------------------   CBL-20
-------------.S-------------------------I-------   ISY
----------------------------I--------------------   ST
-----------I-----------------------------------   BEN
```

Figure 4.6. Amino acid sequence alignment of HIV-2 CBL-20 p26 with other HIV-2 strains. Dotted lines indicate where no sequence was obtained for CBL-20.

4.c. Discussion.

Extensive serological cross-reactivity can be demonstrated between HIV-1, HIV-2 and SIV, particularly between the core-proteins which have been characterised and the epitopes mapped using monoclonal antibodies (Minassian *et al*, 1988; Ferns et al, 1989). Previously, thirteen anti-*gag* HIV-1 monoclonal antibodies had been made by immunisation with HIV-1_{CBL-1} (Ferns *et al*, 1987) and mapped using synthetic oligopeptides. The monospecific antibody EH12E1, demonstrated to recognise a confirmational epitope, also exhibited cross-reactivity with HIV-2_{ROD} and CBL-20 when tested by immunofluorescence (Ferns *et al*, 1989). This reagent could therefore theoretically have been applied for the detection of HIV-2 viral antigens in capture assays utilising the cross-reactive epitopes between HIV-1 p24 and HIV-2 p26.

Monoclonal antibodies such as EH12E1 may not provide, however, the most suitable detection reagent since the use of polyclonal antisera raised against recombinant HIV-1 p24 had been proven to be a better choice of reagent for HIV-1 p24 antigen detection in an 'in-house' p24 antigen assay within the Department of Virology. Therefore, rather than optimise an assay using monoclonal antibodies which were not HIV-2-specific, but relied on cross-reacting epitopes, the use of polyvalent human antisera was pursued. Reagents which were more specific for HIV-2 were therefore assessed and developed.

A simple antigen capture assay was established using human polyclonal sera and was demonstrated to detect HIV-2 antigens with the time-course samples from HIV-2 cultures. Viral antigens were clearly detectable at four days in each case, reflected in the reverse transcriptase levels which paralleled the EIA results for CBL-22 and LAV-2_{ROD}. The degree of cross-reactivity of HIV-1 p24 in this assay was very low as demonstrated by the poor reactivity with the HIV-1 time-course samples. By comparison, the Innotest assay was proficient at detecting HIV-1 antigens but reacted only very weakly with the HIV-2 supernatants which were barely detectable even when virus production was highest. Therefore, although cross-reactions within *gag* between HIV-1 and HIV-2 can be demonstrated in serological assays (Chapter 3), these may only be weak in some cases although strong in others and therefore unreliable and inappropriate for exploitation by diagnostic assays.

The greater homology between HIV-2 and SIV, however, particularly within *gag*, has allowed detection of both HIV-2 p26 and SIV p27 capsid antigens with a single assay (Lairmore *et al*, 1993) and the development of antigen capture assays capable of detecting both HIV-2 and SIV (Thorstensson *et al*, 1991). Furthermore, an SIV p27 antigen assay has been used to measure HIV-2 antigens in the supernatants of cultured cells and plasma derived from HIV-2-infected patients (Simon *et al*, 1993). The antigenic cross-reactivity of SIV p27, for which a commercial assay was available was found to be adequate for this purpose. Considering the relative inefficiency of at least one commercial assay claiming to be capable of detecting HIV-2 antigens (ie the Innotest assay) this was a prudent choice and the cross-reactivity between HIV-2 and SIV core proteins would appear to be sufficient to achieve this.

However, no studies have been published describing the detection of p26 antigen directly in patients' serum, as an indication of virus replication and viral load, as has been applied for HIV-1-infected individuals and was not attempted in the current studies. It has been demonstrated that the prevalence of circulating HIV-1 p24 antigen in African AIDS patients is considerably lower than in European patients at a similar stage of disease (Kaleebu *et al*, 1991). Given that the overall proportion of patients with symptomatic disease in HIV-2 infection is also likely to be lower than in HIV-1, the significance of a negative antigen result would be difficult to interpret. It was also becoming clear that the pursuit of PCR-based methods to measure virus load was likely to be more sensitive than antigen assays. The establishment of sensitive, specific qualitative and quantitative PCR assays for HIV-2 was therefore viewed as the more pressing objective and greater emphasis placed on developing such technologies in preference to antigen-based assays.

However, PCR-based techniques were initially applied to the production of a recombinant p26 protein which was intended for use in the development of an HIV-2-specific p26 antigen assay. CBL-20, was used to make a recombinant GST-p26 fusion protein using the pGEX-3X expression vector which directs the synthesis of foreign polypeptides after fusion with the C-terminus of Sj26, a 26-kDa glutathione S-transferase enzyme encoded by the parasitic helminth *Shistosoma japonicum*. GST-p26 fusion proteins produced using this system were purified from crude bacterial lysates by affinity

chromatography on immobilised glutathione using one of two purification regimes. The plasmid vectors have been engineered such that the GST carrier can be cleaved by digestion with a site-specific protease, using either thrombin (pGEX-2T) or blood coagulation factor Xa (pGEX-3X) as used in these studies. A whole series of these vectors are now available through commercial sources.

The synthesis of fusion proteins is under the control of an IPTG-inducible *tac* promoter and the normal termination codon of GST has been replaced by a polylinker containing unique recognition sites for *Bam*H1, *Sma*1 and *Eco*R1 restriction endonucleases, followed by translation termination codons in all three reading frames. This potentially allows any gene fragment to be expressed as a GST-fusion protein. The proteins produced are generally soluble, as was the case with CBL-20 protein, and of relatively high purity. The system offers a relatively simple means of deriving a recombinant antigen if a bacterial product is adequate. The anti-p26 serum and p26 antigen produced, however, are important reagents and may be applied in future collaborative studies. Alternative reagents are also available, such as SIV p27 *gag* proteins which have also been expressed in *E.Coli* as a soluble *lacZ*-p27 fusion protein and also purified by affinity chromatography (Almond *et al*, 1990) and could be applied in studies of HIV-2.

The *gag* gene of HIV-1 has also been used as a target for DNA sequence analysis to determine the relative phylogenetic relationships between various strains of HIV-1, from different geographical areas of the world (Louwagie *et al*, 1993). Of the three structural genes of HIV-1 (*gag, pol* and *env*) *pol* is most highly conserved, whereas *env* is the most variable. HIV-1 *gag* genes are approximately 75% conserved between different isolates and a similar degree of genetic variation can be found within HIV-2/SIV *gag* sequences (Myers *et al*, 1992). Comparison of the nucleotide sequence of the coding region of CBL-20 expressed in pGEX to the other Gambian isolates (CBL-21/22/23) indicated although there were sequence changes both between these four isolates and compared with the prototypic strain HIV-2_{ROD}, these isolates were all similar to other HIV-2 subtype A viruses according to their *gag* sequence. Translation of the nucleotide sequence of CBL-20 into the amino acid sequence and alignment with other HIV-2

subtype A viruses indicated a high level of conservation at the protein level. The CBL-20 protein is therefore representative of other previously described HIV-2 isolates.

It was initially planned to extend the sequencing studies to determine the relative genetic variability of HIV-2 strains in The Gambia by comparing *gag* sequences. However, although amplification of the p26 gene was possible in most cases from PBMC-extracted DNA, the direct sequencing of bulk PCR product from patient material proved problematical. The limited sequence data that was generated indicated similar synonymous and non-synonymous substitutions to be present in clinical samples and were mostly the same as those described for the CBL strains although detailed phylogenetic studies of HIV-2 strain variation *in vivo* was not pursued.

Some sequences were derived from patients with low proviral DNA loads as described in Chapter 5, but rather than sequence all amplified material in this way, a more rapid approach to genetically identifying HIV-2 strains was pursued and a PCR-based genotype approach to HIV-2 strain identification developed. The LTR, rather than *gag*, was used for this and a strategy for HIV-2 strain subtype identification based on LTR U3 sequences is described in Chapter 6.

Chapter 5.

HIV-2 proviral DNA detection and quantification.

5.a. Introduction.

Advances in molecular technnology have in recent years played an increasing role in the diagnosis and study of viral infections. One of the most significant advances has been the application of the polymerase chain reaction (PCR) which selectively amplifies segments of DNA through a series of denaturation, annealing and extension reactions which are primed by specific oligonucleotide sequences and catalysed by thermostable DNA polymerases (Saiki *et al*, 1988). The applications of PCR are numerous and the greater sensitivity of this method compared to more conventional techniques lends itself to the detection of viral genome directly in clinical samples. This powerful technique can be applied to the detection of rare gene sequences present amongst a background of cellular DNA or host protein and the studies described in this section deal specifically with the application of PCR-related techniques to the detection and quantification of HIV-2 genomes *in vivo*.

In particular, nested PCR has been employed which is a modification of the basic reaction. Two separate rounds of PCR are performed with the product of the primary reaction serving as a template for the secondary reaction (Mullis and Faloona, 1987). Both single and nested PCR assays have been used for the detection HIV sequences in clinical specimens (Ou *et al*, 1988; Simmonds *et al*, 1990). Since a DNA template is required for amplification, only integrated HIV proviral DNA is directly detectable. Amplification of cell-free virus in either the plasma or serous fraction of venous blood is also possible although this first requires a reverse transcription step to produce a complementary DNA (cDNA) copy (Zhang *et al*, 1991). A number of applications of PCR have been envisaged with regard to HIV infection. In certain situations, detection of an antibody response may be either very difficult to interpret or inappropriate to measure. Where dual infection with both HIV-1 and HIV-2 may be suspected on the grounds of strongly cross-reactive antibody between the two viruses, PCR can be applied to attempt to resolve such reactivity. In the case of infants born to HIV-seropositive mothers, PCR may be used to detect viral genomes where the interference of maternal

antibody in serological assays may preclude the identification of vertical transmission. In both cases, the direct detection of viral genome may clarify such issues.

The application of PCR to HIV, however, has particular problems associated with it, the principal consideration being the relatively wide genetic variability of HIV strains. Since HIV exists both as an integrated provirus and as cell-free exogenous virus, the detection and measurement of these two biologically distinct forms of the virus is important. Plasma viraemia levels in HIV-1-infected individuals have been measured in order to monitor anti-viral chemotherapy by the quantification of virus load from the co-cultivation of plasma or cells (Coombes *et al*, 1989; Ho *et al*, 1989). PCR can also be used to provide quantitative and semi-quantitative measurements of the amount of virus present in infected individuals and has now been extensively applied in this field, with commercial assays now available for HIV-1 genome detection and quantification. In contrast, relatively few studies relating to the detection of HIV-2 genomes in clinical samples using PCR assays have been performed and studies of viral load in HIV-2-infected individuals are also scarce.

The aim of the work described in this Chapter was to establish a set of oligonucleotide primer sequences designed for maximal sensitivity which could be applied under optimal conditions for the detection of HIV-2 proviral DNA sequences in clinical material. A number of regions of the HIV-2 genome were selected for amplification of HIV-2 strains using computer searches of known sequences in the database and where possible to also detect SIV-related strains including SIVmac and SIVsm. The development of a quantitative radiometric incorporation assay for the quantification of HIV-2 proviral DNA load was also pursued and the relationship between HIV-2 cellular load and the immunological status of the patient was investigated.

5.b. Results.

5.b.1. Detection of HIV-2 proviral DNA *in vivo*.

i. Establishment of HIV-2 nested PCR.

Nested PCR primer pairs were evaluated for their ability to detect HIV-2 proviral DNA and to determine the prevalence of HIV-2 genome in individuals identified as being anti-HIV-2 positive. The initial reactivity of each primer set was determined using relatively high concentrations of purified DNA (0.5-1μg) extracted from laboratory strains of HIV-1, HIV-2 and SIVmac-infected cell lines prepared as described in Chapter 2.b.2.i. and the four Hirt DNA preparations (CBL.20-23). DNA extracted from uninfected cell lines were used as controls.

Serial ten-fold dilutions of CBL-20 Hirt DNA and CBL-22 DNA extracted from chronically infected C8166/CEM cells were used to demonstrate PCR end-point detectability for *vpx* and LTR primers. The UNIPOL primers were tested on HIV-2 and HIV-1 (HTLV-III/H9) DNA in a hemi-nested PCR (UNIPOL primers 2 and 3 in primary amplification and UNIPOL 1 and 2 in secondary amplification). UNIPOL primers reacted strongly with HIV-1, HIV-2 and SIVmac DNA. *Vpu* and *vpx* primers were demonstrated to have good specificity with HIV-1 and HIV-2 DNA from laboratory isolates respectively, although *vpx* primers failed to react with SIVmac DNA even at relatively high (>1000) copies. LTR and *vpx* nested primers were therefore demonstrated to be HIV-2-specific although of the three sets capable of HIV-2 DNA detection (LTR, *vpx*, UNIPOL), the LTR primers gave the strongest signals in early attempts to amplify HIV-2 DNA from HIV-2-infectected (seropositive) individuals

A comparison of primary and secondary amplification reactions with LTR primers is shown in Figure 5.1. with DNA extracted from eight anti-HIV-2 positive individuals and ten uninfected, seronegative individuals as determined by HIV-2-specific competitive EIA (Chapter 3). DNA was extracted by phenol/chloroform and 0.5μg (determined by spectrophotometry) used for each PCR. PCR assays in general were performed using 35 heat cycles (first round) and 25 heat cycles (second round) for all primer sets in comparisons of primer reactivity. Non-specific priming of cellular DNA was observed, although HIV-2-specific bands were not visible in primary PCR with clinical specimens.

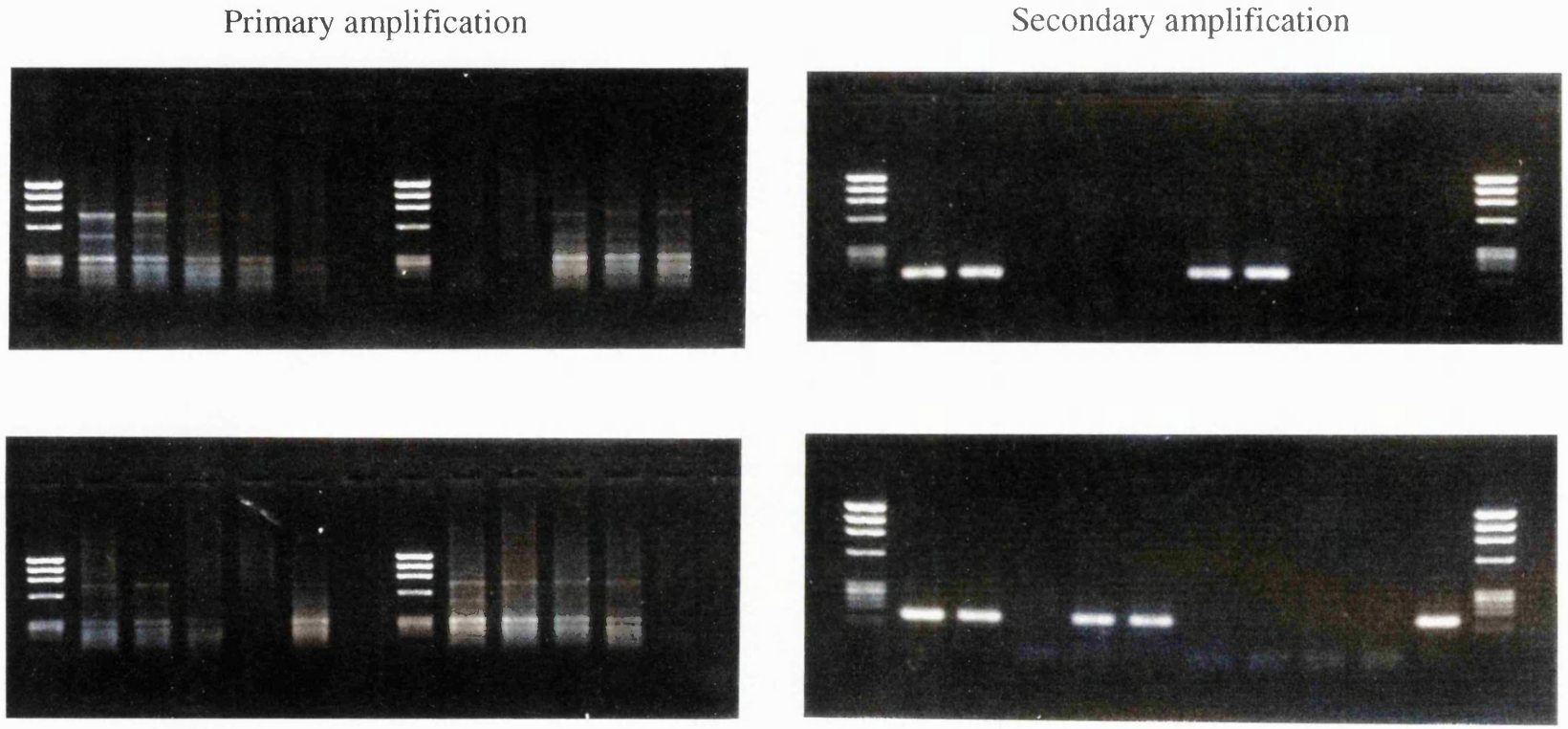


Figure 5.1. Primary and secondary amplifications performed with the diagnostic LTR primer set. HIV-2-specific DNA is visible only upon secondary amplification in samples from eight HIV-2-infected individuals compared with those from ten uninfected individuals as a single band of 141bp. Molecular weight marker = ØX174 digested with *Hae* III CBL-20 Hirt DNA (100 copies) was used as the positive control, final track lower gel.

Secondary amplification yielded high intensity HIV-2-specific amplicons of the calculated, theoretical molecular weight (Figure 5.1.).

Preliminary studies relating to magnesium and primer concentrations were performed although in practice reaction conditions commonly reported in the literature yielded high intensity bands upon secondary amplification as visualised by agarose gel electrophoresis and ethidium bromide staing. Magnesium ion concentrations of 1.5-2mM were used in all further experiments. Primer concentration was assessed using 10 copies of CBL-20 DNA and 10 picomoles of primer found to provide efficient amplification although in practice primer concentrations of 5-20 picomoles also yielded high intensity bands. Using calculations based upon the theoretical melting temperature (Tm) of each primer, an anneal temperature in the primary reaction of 55°C was used followed by a secondary reaction anneal temperature of 50°C. Temperatures of 55°C and 60°C were compared in the secondary amplification with no advantage in terms of specificity gained with the higher temperatures

ii. PCR reactivity with DNA extracted from Gambian HIV-2-infected individuals.

The relative reactivities of the LTR, UNIPOL and *vpx* primer sets were compared using a panel of DNA samples extracted from a cohort of HIV-2 seropositive and seronegative prostitutes from the Faraffeni region of The Gambia, classified according to the serological criteria already described (Chapter 3). The results of these experiments are summarised in Table 5.1. Cellular DNA in these experiments was extracted and purified by conventional phenol/chloroform extractions and these samples were also tested with the *vpu* primers in these early studies. Cellular DNA concentrations were determined by spectrophotometry due to the relatively pure DNA content following ethanol precipitation. Yields varied from several up to 50μg of total DNA and between 0.5-1μg of each sample was used per PCR.

The results obtained with the LTR, UNIPOL, and *vpx* primer sets were instructive in the application of nested PCR to DNA extracted from primary, uncultured PBMCs. The *vpu* primer set was unreactive with all DNA samples tested in this series of samples, except RE231 which amplified with LTR, UNIPOL *vpu* and *vpx* primers. The LTR primer set

Sample	HIV-2 C-EIA	Target region for PCR amplification			
		LTR	*vpx*	UNIPOL	*vpu*
RE 58	-	-	-	NT	-
RE 67	-	-	-	NT	-
RE 74	-	-	-	NT	-
RE 76	+	++	+	++	-
RE 82	-	-	-	-	-
RE 83	-	-	-	NSp	-
RE 86	-	-	-	NT	-
RE 92	-	-	-	-	-
RE 93	+	++	++	++	-
RE 94	-	-	-	-	-
RE 99	+	++	++	++	-
RE 120	-	-	-	NT	-
RE 124	-	-	-	NT	-
RE 137	+	++	-	-	-
RE 145	+	++	++	Nsp	-
RE 146	+	++	++	Nsp	-
RE 151	-	-	-	NT	-
RE 213	+	++	++	Nsp	-
RE 220	+	++	++	NT	-
RE 221	+	++	-	-	-
RE 232	+	++	-	-	-
RE 188	+	++	+	++	-
RE 231	+	++	++	++	+

Table 5.1. Reactivity of LTR, UNIPOL, *vpx* and *vpu* primer sets with DNA samples from the Faraffeni cohort.

NT = Not tested.
NSp = Non-specific reaction, characterised by spurious amplification from both HIV-infected and uninfected individuals.
Patient RE231 represents dual HIV-1 and HIV-2 infection by *vpu/vpx* PCR and was serologically strongly dually reactive by competitive EIA.

provided the most consistent results and exhibited 100% concordance with serological findings. In each case, high intensity bands were obtained with this primer set compared with lack of amplification with negative samples. All amplified fragments were of the same, expected size (141bp) as determined by agarose gel electrophoresis and ethidium bromide staining. By comparison, the *vpx* and UNIPOL primers yielded different amplification efficiencies. The *vpx* samples reacted weakly with some samples, though were clearly HIV-2 specific, and failed to detect three samples positive by LTR primers. The UNIPOL primers also produced a varied response. Specific bands of the predicted molecular weight were identified with some DNA preparations, although not others, which were also positive with the LTR primers, although non-specific bands which were both of lower and higher molecular weight than the expected band-size were also produced with some samples. Comparisons of amplification of DNA with LTR and UNIPOL primers from five HIV-2-infected and five HIV-2-uninfected individuals is shown in Figure 5.2. exemplifying these effects.

One of the potential problems with the UNIPOL primer configuration was the lack of a fourth primer. The non-specificity of this primer combination is most likely the result of priming by endogenous *pol* sequences or the non-specific priming by cellular DNA. These sequences were re-designed to allow a fully nested approach for HIV-2 and SIVmac/SIVsm strains and the option of amplifying HIV-1 strains with these primers dispensed with. This was successfully achieved and the primers used are described in Table 2.1. Although the 3' end of the *pol* gene is highly conserved some variation exists and neutral inosine residues were incorporated where there was no overall consensus. These primers were subjected to the same process as described above and then reacted back on the Faraffeni DNA samples. Excellent concordance (100%) was observed between the LTR and *pol* primers.

iii. Primer specificity.

The specificity of the nested PCR primer sets for HIV-1 and HIV-2 was further tested by comparing reactivity with a range of DNA extracts obtained from laboratory isolates for HIV-1, HIV-2 and SIV. The results are shown in Figure 5.3. Good overall specificity was demonstrated using 1μg of DNA with no primer set showing cross-amplification. The *vpu* primers, however, did not amplify with U455a or Z84, both of which are

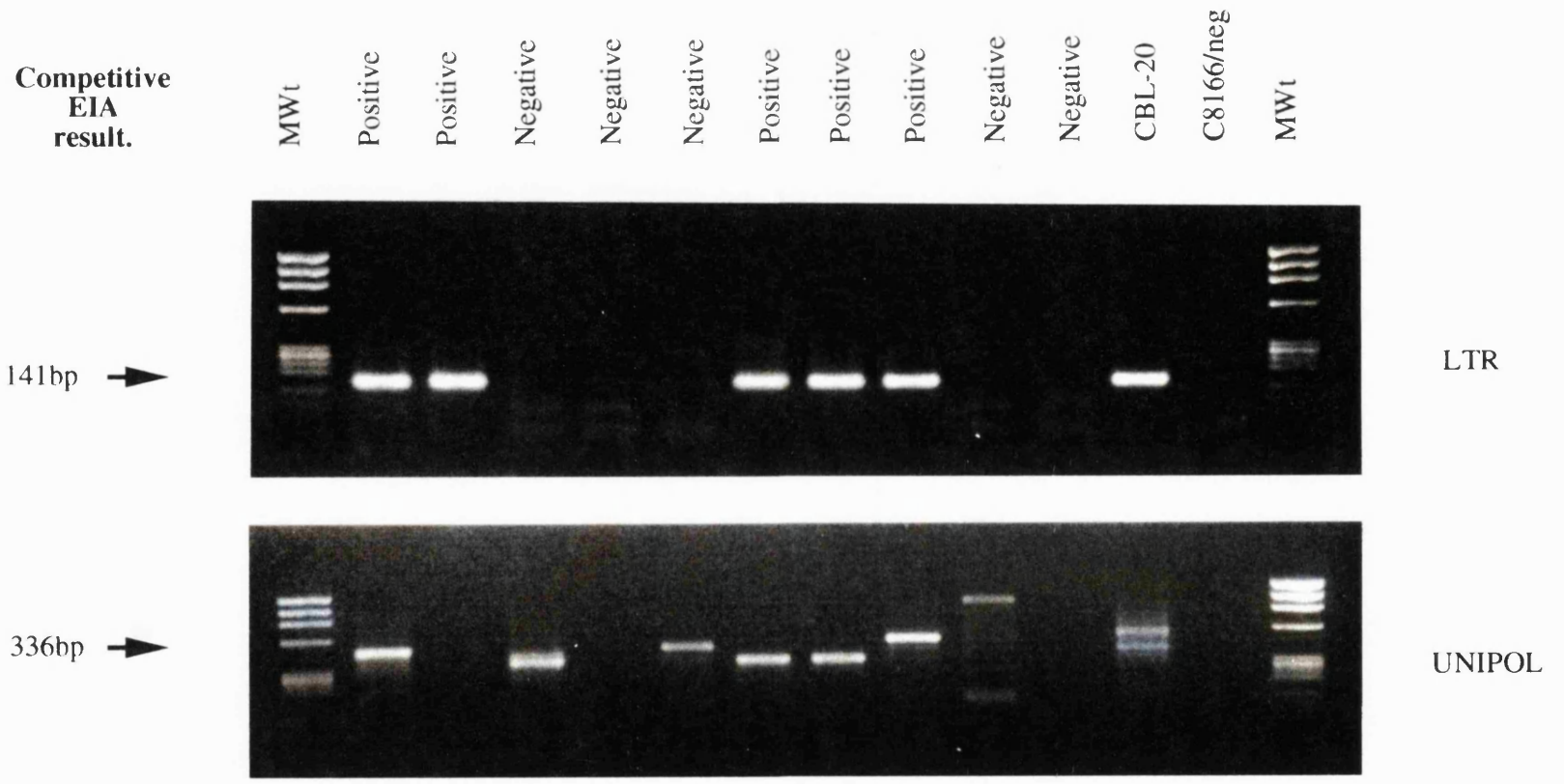


Figure 5.2. Secondary PCR amplification of HIV-2-infected and uninfected individuals using LTR and UNIPOL primer combinations. Spurious satellite bands were generated with the UNIPOL but not with the LTR primer set. The CBL-20 control DNA appeared as a doublet with UNIPOL primers. Molecular weight marker = ØX174 digested with *Hae* III.

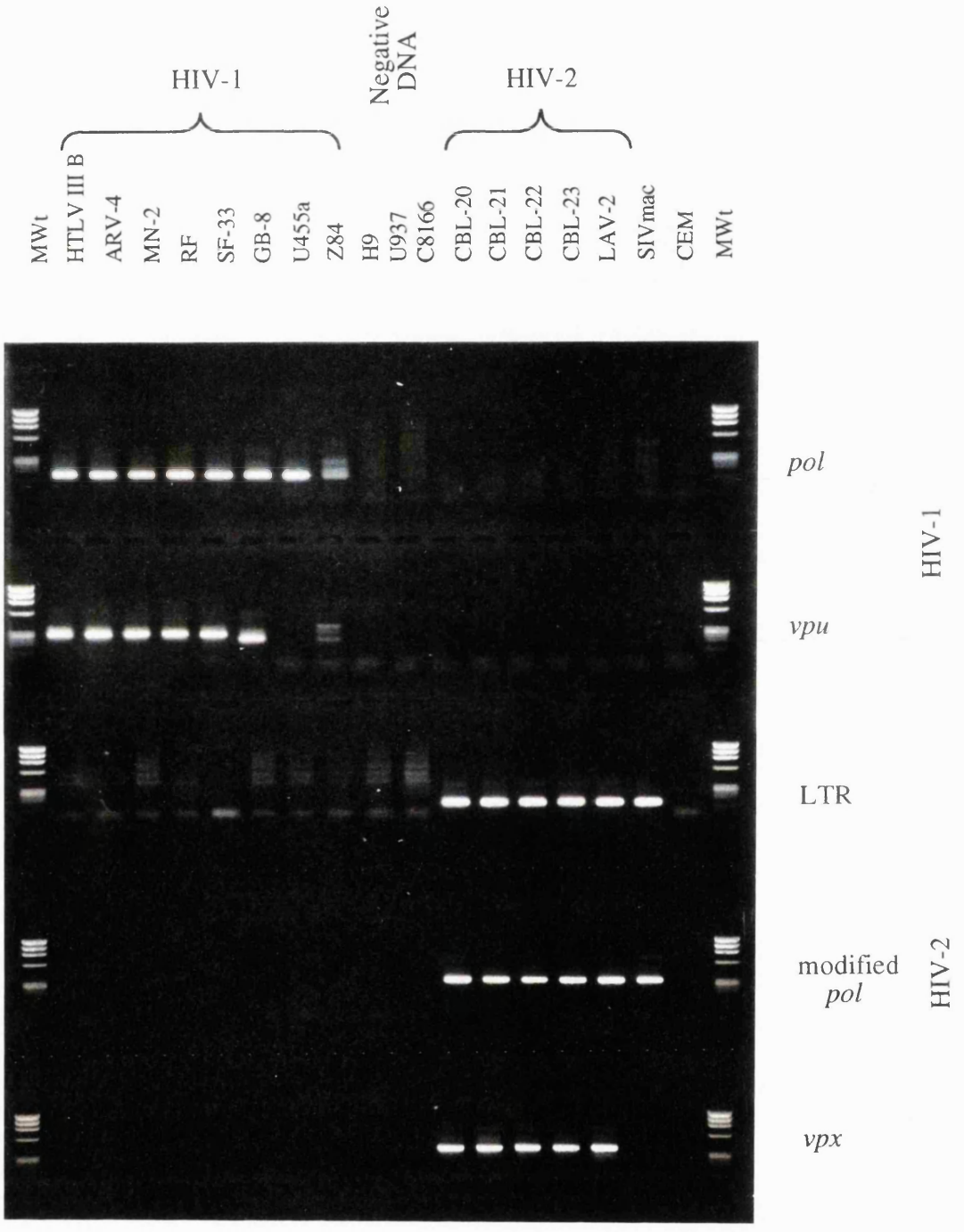


Figure 5.3. Specificity of HIV-1 and HIV-2 nested PCR primer sets on a panel of HIV-1, HIV-2, SIV and negative DNA preparations.
Molecular weight marker = ØX174 digested with *Hae* III. Predicted band sizes are listed in Table 2.1. and Table 2.2.

African strains. The *vpu* primer set also exhibits a deletion with strain GB-8. The HIV-2 primer sets showed no cross-reactivity with the heterologous or negative DNA samples. LTR, modified *pol* and *vpx* primer sets were also demonstrated to have good specificity, although the *vpx* primers failed to react with the SIV DNA preparation even at relatively high copy number. The net result of these preliminary studies therefore, was the development of two nested primer sets (two pairs for each region) in the LTR and *pol* gene, both representing two putatively highly conserved regions of the genome.

iv. Comparitive sensitivity of LTR primers with HIV-2 and SIV DNA.

To determine the sensitivity of the LTR primer set with HIV-2 and SIV DNA, end-point dilution studies were performed. Serial ten-fold dilutions of DNA samples from both viruses were reacted with LTR primers and the approximate end-point determined. Purified CBL-22 and SIVmac DNA derived from cultured virus and extracted using phenol/chloroform were used. DNA corresponding to the final dilution signalling by nested PCR for each was further diluted to demonstrate single molecule amplification as described in 2.b.4.ii. The reciprocal dilution for CBL-22 (3/4 negative bands) relates to 1.4 copies per µl of DNA, equivalent to 14 copies per 10µl. For SIV DNA, 1.4 copies per µl was also obtained with 1/4 and 3/4 negative bands for dilutions of 1 and1/5 respectively.

A Poisson distribution of end-point molecules by limit dilution studies was demonstrated on SIV and CBL-22 DNA as shown in Figure 5.4. where less than 20% of amplified reactions signalling positive is taken to represent single molecule amplification on the basis of probability. From comparisons of the database, sequence homology of HIV-2 and SIV were high in these regions and this result could therefore perhaps have been predicted although demonstration of this by such experiments was informative. The Poisson formula was used to establish the number of proviral DNA copies as the detectable end-point of nested PCR in control and patient samples in further studies.

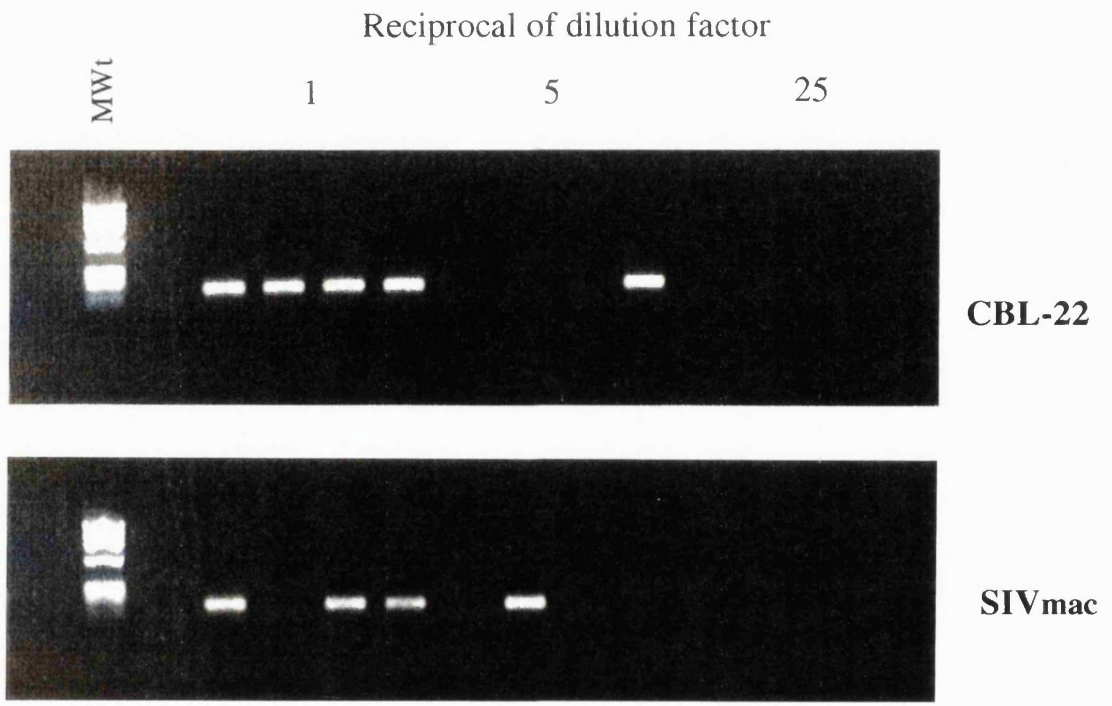


Figure 5.4. Limit dilution of HIV-2 and SIV DNA amplified with the LTR primer set. According to the distribution of negatives and calculated by the Poisson formula, the CBL-22 and SIVmac samples contain 1.43 and 1.40 molecules per µl equivalent to approximately 14 copies/molecules per 10µl of DNA input.

v. Nested PCR with rapid-preparation DNA method.

The LTR and *pol* primer sets were subsequently applied to DNA preparations extracted from clinical samples. The *vpx* primers were not used in these studies. It was anticipated that phenolic extraction of DNA on a large number of patient specimens was not a practical proposition if relatively large numbers of patient specimens were to be tested in London or The Gambia. A protocol for rapid and convenient extraction of DNA was evaluated, initially with the LTR primers and subsequently on a sub-set of samples with the modified *pol* primers.

A total of 101 DNA extracts, prepared by the rapid protocol method (section 2.b.2.iii) were tested from 86 patients for the presence of HIV-2 DNA sequences using the LTR primer set. Initially, 41 samples were tested by LTR primers. The sensitivity of the LTR primers for the detection of HIV-2 proviral sequences was 97% (40/41). Thirty samples from seronegative individuals were tested in parallel and all were negative. LTR primers were tested on a further 60 DNA preparations from an additional 45 patients in The Gambia confirming the sensitivity to remain high at 97% (84/86). The two patients in whom HIV-2 DNA was undetectable had relatively high CD4 counts (>500cells/mm^3). The initial set of 41 samples were also tested for *pol* sequences which were not detected in two samples including one of the samples which failed to amplify with the LTR primer set giving a sensitivity of 95% for *pol* primers (39/41). Fifteen pairs of samples came from sampling at two separate times. There was complete concordance between the results of the first and second analysis, since all patients that were initially found to carry HIV-2 LTR sequences gave the same results when the later samples were assayed.

The prevalence of HIV-2 genome detection was therefore demonstrated to be high with both primer sets and confirmed the use of such a rapid extraction protocol was viable for screening large numbers of patient samples HIV-2 provirus detection. The results obtained with this larger collection of PBMC samples were therefore similar to those obtained with the phenol/chloroform-extracted samples when compared with the serological status of the individual. The use of fluorometry was found to be a convenient means of assessing the total cellular DNA concentration within the sample for adjusting the DNA concentration for PCR testing.

vi. Investigation of dual serological reactions by PCR.

The use of HIV-1 and HIV-2 PCR assays was also applied to samples from some individuals with strong reactivity in HIV-1 and HIV-2 competitive EIAs. The number of these individuals was relatively low representing a very small proportion of HIV-infected individuals in The Gambia. As a result the number of PBMC preparations from which DNA was available in these early studies was also low. From the serological profiles obtained with the Farafenni samples, two individuals (RE188, RE231) were identified as having antibody reactivity compatible with dual infections. The *vpx* primers had been evaluated on the set of DNA samples from the Farafenni cohort as described in 5.b.1.ii. Similarly, the *vpu* primers were tested with a set of 44 DNA samples from HIV-1-infected individuals in London, which had previously been tested with HIV-1 *pol* primers (Table 2.2). However, only 40 were detectable by *vpu* PCR, all of which were positive by HIV-1 *pol* primers (40/44 = 90% sensitivity). Therefore both the *vpu* and *vpx* primer sequences failed to detect either HIV-1 or HIV-2 genomes which were detectable with other primer sets. However, early applications of the *vpu/vpx* primer set with the Farafenni samples indicated one individual with dual *vpu/vpx* primer reactivity. Figure 5.5. demonstrates co-infection of patient RE 231 with dual PCR reactivity with these primer sets. Both RE231 and RE188 were positive with hemi-nested UNIPOL primers, though RE188 was *vpx* positive but *vpu* negative (Table 5.1).

A further set of samples originally classified as containing anti-HIV-1 (W series) were tested by HIV-1 *pol* and HIV-2 LTR nested PCR but not by *vpu/vpx* PCR. Eight were positive with the HIV-1 *pol* primers, one was HIV-2 LTR positive and one was positive with both primer sets. To demonstrate the potential difficulties and differing reactivities of serological and PCR testing, the combined results of twelve samples from seronegative, HIV-1 seropositive, HIV-2 seropositive and dually seroreactive patients including RE188 and RE231 are summarised in Table 5.2. The PCR reactivity of these, including the W series samples is shown in Figure 5.6. These data indicate that there is not in fact complete agreement between the serological and PCR results, highlighting the difficulties of HIV diagnosis and the speciation of these viruses. Sample P1121 was identified as containing anti-HIV-2, although the level of reactivity when normalised optical densities were used was lower than obtained with other samples which contained

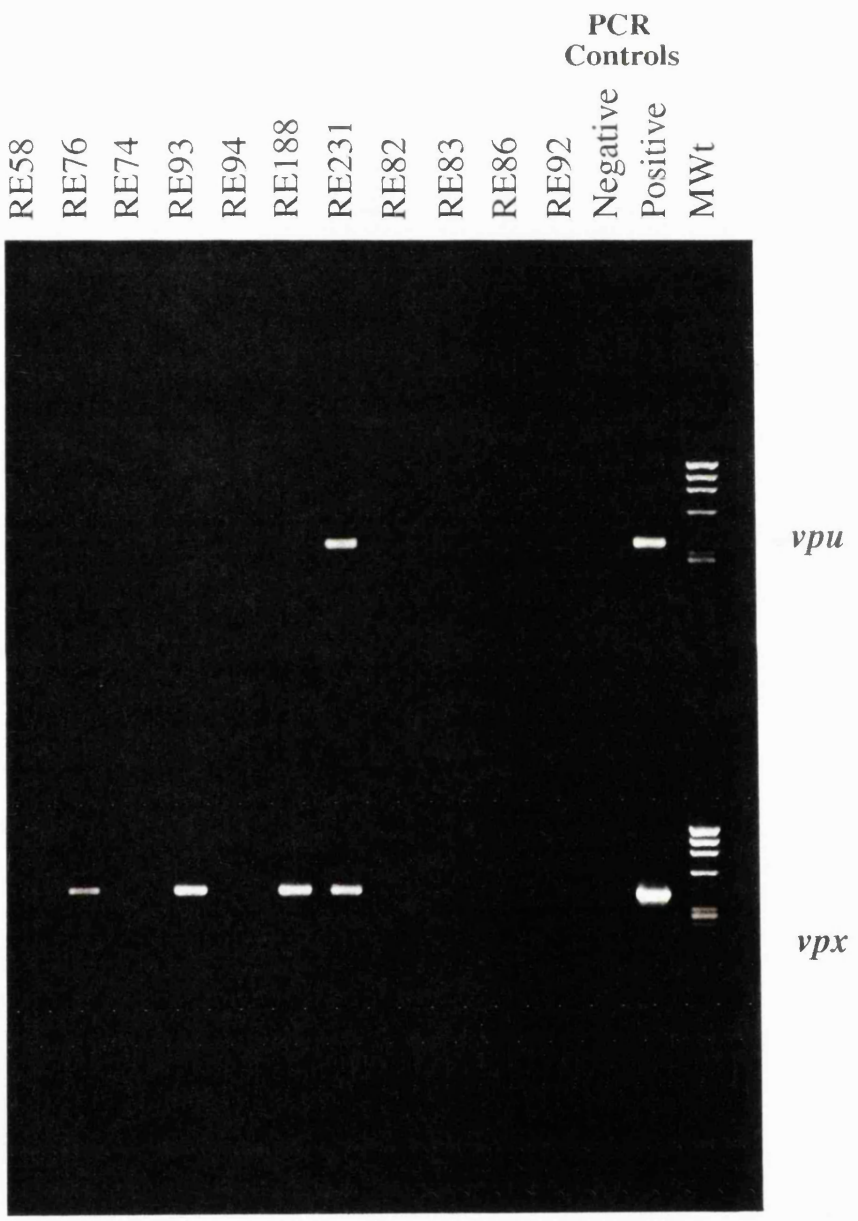


Figure 5.5. Reactivity of nested HIV-1 *vpu* and HIV-2 *vpx* primers.
DNA was amplified from samples from HIV-infected and uninfected individuals. Sample RE231 shows dual *vpu/vpx* PCR reactivity. Samples RE 76, RE 93, and RE188 were reactive with *vpx* primers only. CBL-1 and CBL-20 DNA were used as HIV-1 and HIV-2 controls respectively.

Sample	Competitive EIA[a]		PCR result[b]	
	HIV-1	HIV-2	HIV-1	HIV-2
H 1829	<1	23.2	-	+
W 1536	NA	NA	+	+
W1713	61.6	<1	+	-
W1694	40.1	<1	+	-
P 1121	<1	3.65	-	-
P 1160	7.9	1.12	+	-
P 1435	<1	12.4	+	-
P 1461	<1	15.7	-	-
P 977	9.49	28.4	+	+
P 103	129.5	68.2	+	+
RE 188	16.0	39.9	+	+
RE 231	14.98	20.2	+	+

Table 5.2. PCR and serological reactivity of possible dually infected and uninfected individuals.

[a] Normalised optical density values were used for comparative purposes.

[b] Nested HIV-1 *pol* and HIV-2 LTR primers were used.

NA =Not available whereby no serological results were obtained for the date of the sample tested by PCR. Serological results obtained over 2.5 years prior to this indicated HIV-1 infection only for sample W 1536.

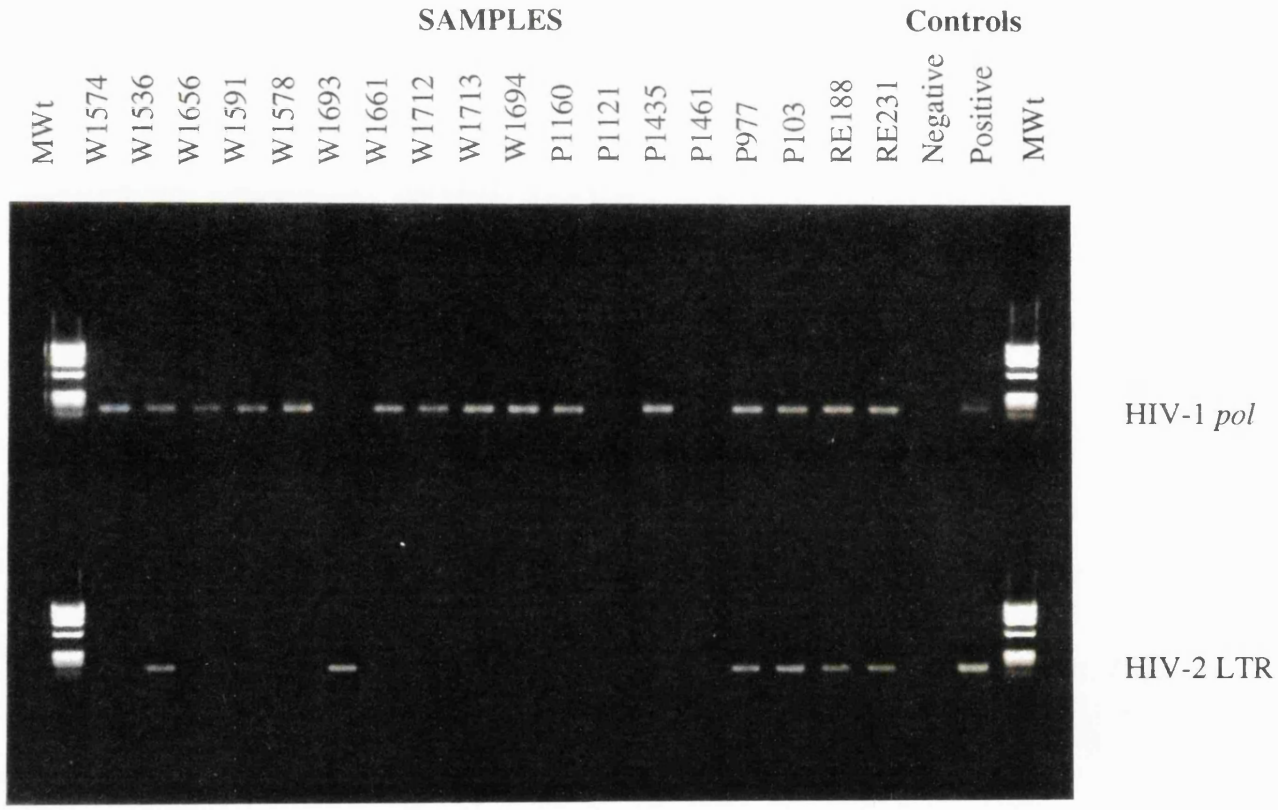


Figure 5.6. Reactivity of nested HIV-1 *pol* and HIV-2 LTR primers with clinical samples. Dual HIV-1 and HIV-2 PCR reactivity was obtained for five samples (W1563, P977, P103, RE188, RE231).

generally higher levels of levels of anti-HIV-2. A normalised OD of 3.65 and a negative PCR result in this patient may therefore be a reflection of low level specific reactivity or non-specific reactivity in the anti-HIV-2 assay. Re-bleed samples were not available from this patient for further analysis. Patient P1461 had high levels of anti-HIV-2 (normalised optical density of 12.4) but remained repeatably PCR negative. This patient represents one of the few samples who by all serological criteria imposed was repeatably and strongly positive for anti-HIV-2 and therefore considered to be infected with HIV-2, but in whom HIV-2 genome was undetectable. In these circumstances it is also important to exclude HIV-1 by both antibody and genome detection. In the case of sample P1435, by serology this would be classified as containing anti-HIV-2 only although by PCR HIV-1 genome was detected by nested PCR but not for HIV-2. Discordant serological and PCR results were identified, therefore but were the exception rather than the rule in the samples analysed. In these circumstances, it is important to gain a second sample for confirmatory purposes although considering the potential difficulties in patient follow-up this was not always possible.

Dual infection would only be defined where independent amplification of both genotypes from the the same DNA preparation was demonstrable. Due to the differences in the biological specificity of DNA and antibody detection, a dual HIV-1 and HIV-2 PCR result may be taken as the stronger indication of dual infection. Five such individuals were identified in these studies. These were patient RE231 by *vpu* and *vpx* nested primers and W1536, P977, P103, RE188 and RE231 by *pol* and LTR nested PCR for HIV-1 and HIV-2 respectively. Dual infections were therefore diagnosed in HIV-infected patients in The Gambia. Overall, the agreement between serological testing and the PCR data were good, although more studies of this kind are required.

5.b.2. Quantification of HIV-2 proviral DNA.

A quantitative radiometric PCR assay was developed for use in studies of HIV-2 virus load in London and in The Gambia. Measurements were made on samples which had been previously extracted and tested by LTR PCR. DNA which had been previously used in a primary nested PCR could therefore serve as a template not only for detection purposes but also for quantitative measurements. To achieve this the incorporation of a

radiolabelled nucleotide into secondary amplification reactions, in this instance [^{35}S] labelled dATP, was mediated by modifying the conditions of the secondary reaction.

i. Comparison of *Taq* and *Pfu* DNA polymerases.

Studies performed within the Department on HIV-1 RNA quantification had previously demonstrated that ten cycles of secondary amplification provided a linear, exponential increase in the amount of PCR product generated in incorporation assays (Semple *et al*, 1993). The rate at which this occurs may also be due to the amplification properties of the DNA polymerase used in the reaction. As other DNA polymerases became available through commercial sources, *Pfu* DNA polymerase was compared with *Taq* polymerase in preliminary experiments.

Differences between the two enzymes were demonstrated by parallel amplification with dilutions of pROD10 DNA in c8166 carrier DNA, tested in duplicate, measured by quantititive PCR following 10 heat cycles of amplification as shown in Table 5, Appendix 2. and Figure 5.7. These data clearly demonstrated that *Pfu* is a superior enzyme for this type of reaction when one unit of *Pfu* is compared with one unit of *Taq* polymerase. The response of each enzyme to the same target is also different. A sigmoidal curve with reduced efficiency of incorporation of detectable radiolabel was demonstrated with *Taq* whereas a more linear and expanded response was observed with *Pfu*. The reaction buffer used contains 2mM final concentration of magnesium ions, which is within the optimised range of magnesium concentration for the LTR primer set. Therefore all subsequent optimisation experiments were performed with *Pfu* rather than *Taq*.

ii. Optimisation of qPCR assay.

Modifications to the secondary amplification reactions were performed. The amount of unlabelled nucleotide was reduced in secondary reactions, varied to 40, 20, 10 and 5μM combined with 5μCi of the radiolabel (specific activity, 1400Ci/mmol). Reactions were performed with 10 picomoles of biotinylated primer. Comparison with higher concentrations of primer (25 picomoles per reaction) was found to reduce the signal by approximately half (Table 5, Appendix 2). Therefore the interaction between biotinylated PCR product and streptavidin-coated plates was also assessed by varying the amount of

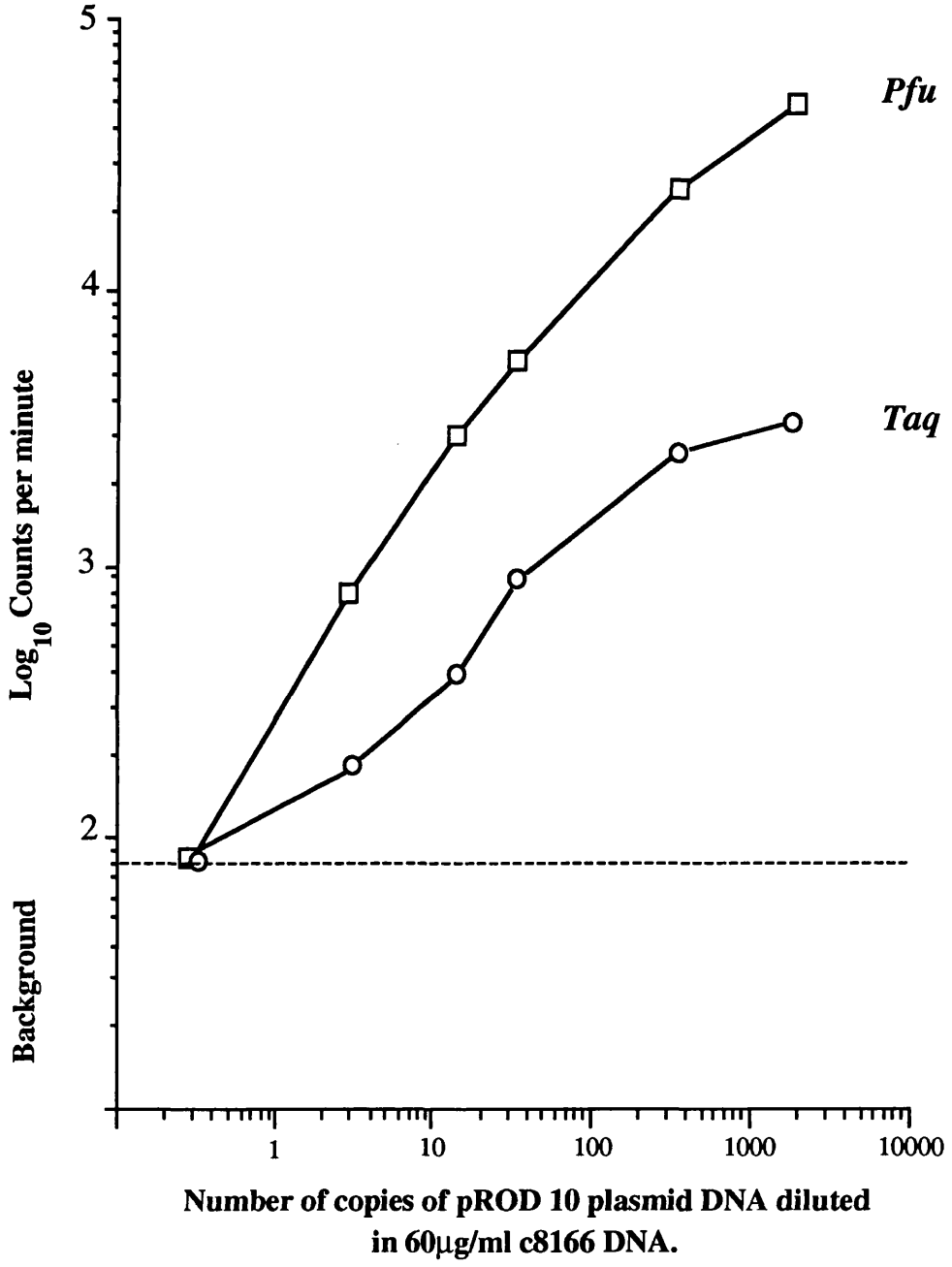


Figure 5.7. Comparison of *Taq* and *Pfu* DNA polymerases using the radiometric quantitative PCR assay. Means of duplicate values were plotted for each dilution.

streptavidin available for capture of the PCR product. Streptavidin was coated at concentrations of 2.5, 5, 10 and 20μg/ml using 10 picomoles of primer per reaction. The results of these experiments are summarised in Figure 5.8. comparing streptavidin concentrations at 2.5 and 5 μg/ml and 5 and 40μM concentrations of unlabelled nucleotide mix. A reduced 'cold' nucleotide concentration of between 5-10μM when used with 5μCi of [^{35}S] dATP produced the widest dynamic range using dilutions of pROD10 plasmid DNA diluted in c8166 negative DNA.

The effect of varying the amount of streptavidin between 5-20μg/ml on the solid-phase was minimal although the lowest copy number (three copies) was undetectable a concentration of 2.5μg/ml combined with 40μM of cold dNTPs. Therefore for all subsequent reactions streptavidin was coated at 5μg/ml. Figure 5.8. also indicates the relatively robust nature of the assay since parallel regression curves produced were reproducible and illustrates the limiting optimal concentration of critical reagents. Combinations of dNTP mix at a concentration of 40μM and 2.5μg/ml of streptavidin were both demonstrated to be sub-optimal.

Final reaction conditions appearing in the protocol were determined to be 5-10μM of each unlabelled dNTP (dGTP, dCTP, dTTP) in conjunction with 5μCi of [^{35}S] labelled dATP with 10 picomoles of biotinylated and unbiotinylated primer using 1 unit of *Pfu* DNA polymerase. PCR product was captured onto wells coated with 5μg/ml of streptavidin, to reduce the costs of this reagent. Using these modifications to the second round PCR reaction it was possible to obtain quantitative measurements from first round amplifications with no further changes to the first round reaction conditions.

iii. Quantification by limiting dilution.

The number of HIV-2 DNA copies present in both control and sample DNA preparations was determined by limiting dilution analysis. This was used both as a means of calibrating the external standard controls in the qPCR assay and to independently determine the number of HIV-2 proviral DNA copies in 40 samples in which HIV-2 provirus was detectable. For these estimations, the LTR primer set only was used.

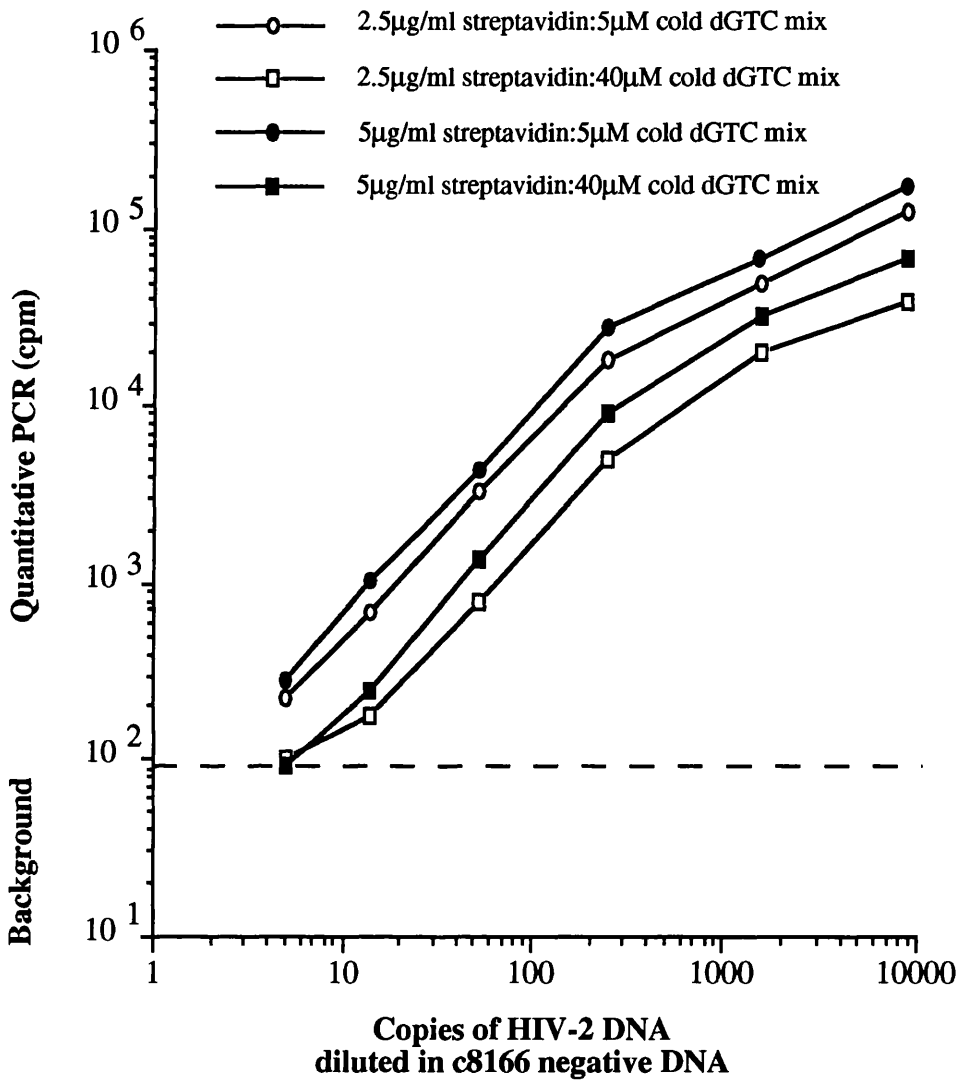


Figure 5.8. Optimisation of streptavidin and nucleotide concentrations in qPCR assay. A constant concentration (5µCi) of radiolabelled dATP was used for each reaction containing 10 picomoles of biotinylated primer. Reduced concentrations of unlabelled nucleotide (dGTC) were compared for incorpoation efficiency using a stardard curve of HIV-2 DNA (pROD 10 plasmid DNA diluted in c8166 DNA). Dashed lines represent the mean background values.

Proviral DNA levels were determined from 10^5 PBMC's by limiting dilution as the number of copies per µl of DNA for each of the 40 DNA samples, the range for which is shown in Table 6, Appendix 2. These data clearly demonstrate that a wide range of proviral DNA levels is present in HIV-2-infected individuals. For validatory purposes, a comparison of the number of copies of HIV-2 proviral DNA amplified from either 10^5 PBMC's or from 10^5 CD4+ lymphocytes obtained with both methods is shown in Figure 5.9. A high level of correlation determined by Spearman rank correlation analysis was observed with 10^5 PBMC's ($r=0.76$, $n=40$, $p<0.001$) and with 10^5 CD4+ lymphocytes an excellent agreement between the two methods was demonstrated ($r=0.93$, $n=40$, $p<0.001$). In the final analyses of clinical data, correlation between the absolute CD4+ count and results based on 10^5 CD4+ lymphocytes were employed.

However, these studies of HIV-2 proviral load initially performed by the more conventional approach of end-point dilution indicate that a broad range of proviral DNA levels is present in HIV-2- infected individuals varying from a few copies per 10^5 PBMCs to several thousand copies 10^5 PBMCs.

iv. Dynamic range of the qPCR assay.

One of the aims when designing assays of this kind is to achieve a good distinction between samples which contain the analyte under investigation and those that do not. The signal produced in a quantitative assay must also be demonstrated to increase in direct proportion to an increase in the amount of analyte in the reaction. From studies of HIV-1 proviral DNA measurements obtained using quantitative assays, a range from several copies to up 10,000 copies (equivalent to 4 X $\log_{10}$ cycles) may be required to assay the entire range of HIV-infected individuals. When establishing the HIV-2 qPCR assay this was a major consideration, particularly the detection of very low (less than ten copies) of HIV-2 provirus. For this purpose the Poisson distribution was employed to calculate statistically the number of DNA molecules present in samples reacting around the lower limits of detectability. This relies upon nested PCR to detect single molecules and is a direct representation of the actual number of DNA molecules amplified.

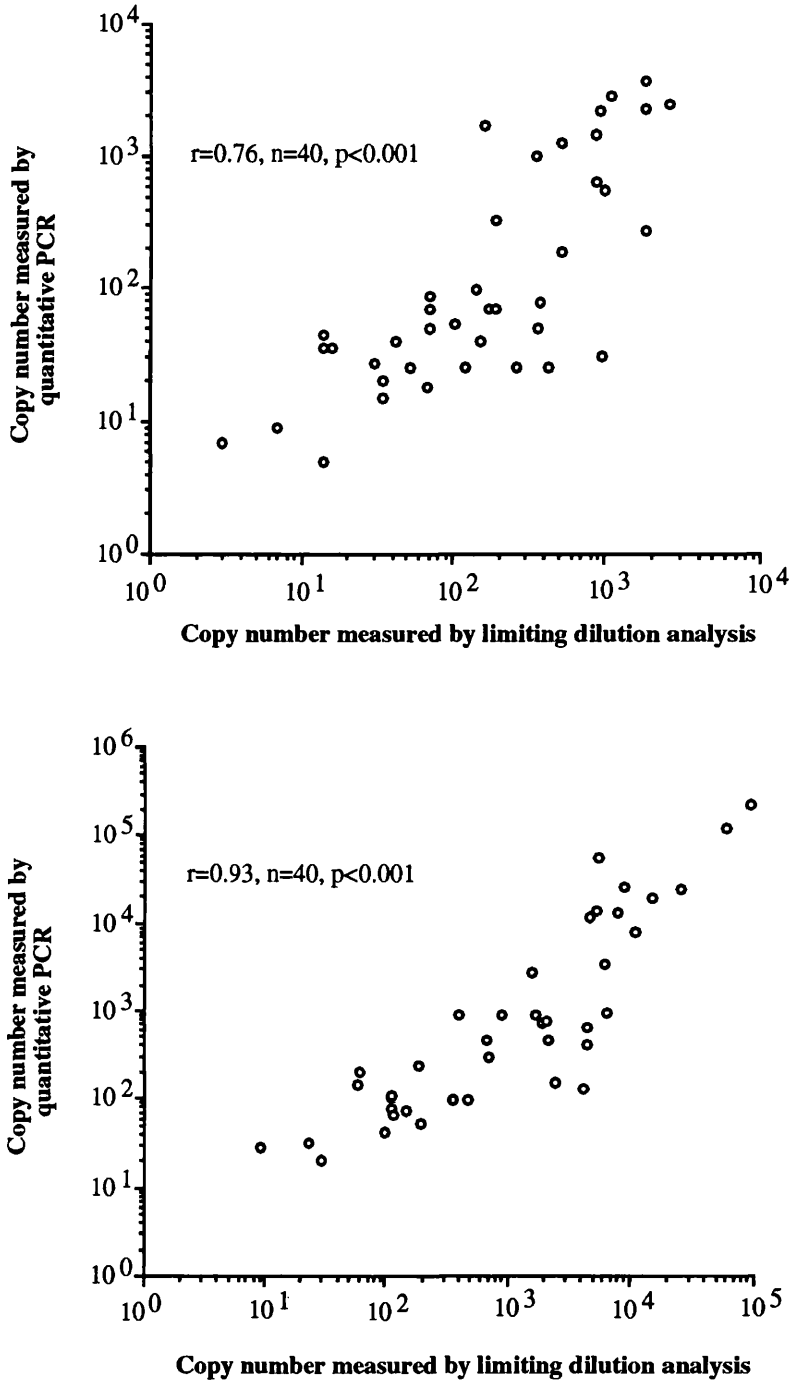


Figure 5.9. Comparison of HIV-2 proviral DNA copy number measured by quantitative PCR and limiting dilution analysis.

Comparison of virus load measured by quantitative PCR assay and limiting dilution analysis expressed as copies per 10^5 PBMCs (upper) and 10^5 CD4 cells (lower). Correlation coefficients are indicated.

The lowest copy number detected with the qPCR assay was three copies, both with pROD plasmid DNA and CBL-22 culture-derived DNA both of which were serially five-fold diluted in 60μg/ml of C8166 negative DNA. In each case the precise number of copies at each datum point was determined by the limiting dilution method. In both cases, single molecule detection capability was demonstrated whereby replicate tubes of the final dilution gave signals by nested PCR on 5/20 and 6/20 occasions with pROD 10 and CBL-22 respectively. Calculation by the Poisson formula indicates that this corresponds to 0.28 and 0.35 molecules per microlitre of DNA respectively, equivalent to 2.8 and 3.5 molecules/10μl. Cut-off values were taken at three times the mean values obtained with the negative samples (80cpm) at 240-250cpm in the qPCR assay.

For practical purposes, however, where amplification of very low copy numbers is a random event, a lower detection limit of five copies was imposed although values relating to lower copy numbers were obtainable. A dynamic range of 5-2000 copies was therefore taken to be the working limits of the assay, over which linearity was demonstrated. Both the plasmid control and CBL-22 DNA diluted in negative carrier DNA were used as standard curves and serial dilutions assessed for HIV-2 copy number proir to use. These were for pROD10: **3, 14, 34, 352,** and **1798** copies per 10μl and for CBL-22: **3, 14, 53, 246** and **1564** copies per 10μl. Stocks of these controls were maintained aliquoted at -20°C and thawed immediately prior to use and unused aliquots discarded. In practice, HIV-2 proviral copy number rarely exceeded 2000 copies per 100, 000 lymphocytes equating to 1/50 infected cells although for some patients higher values were obtained.

It was possible to make comparisons between data generated in London and experiments performed in Fajara, demonstrated by comparison of the two standard curves produced with CBL-22 DNA, showing there was essentially no difference in the data obtained between the two sites (Figure 5.10). This is also an indication of the stability of the assay since reagents had to be transported to The Gambia and the assay established on site. This was the objective of two field visits to the MRC unit during February and November 1993.

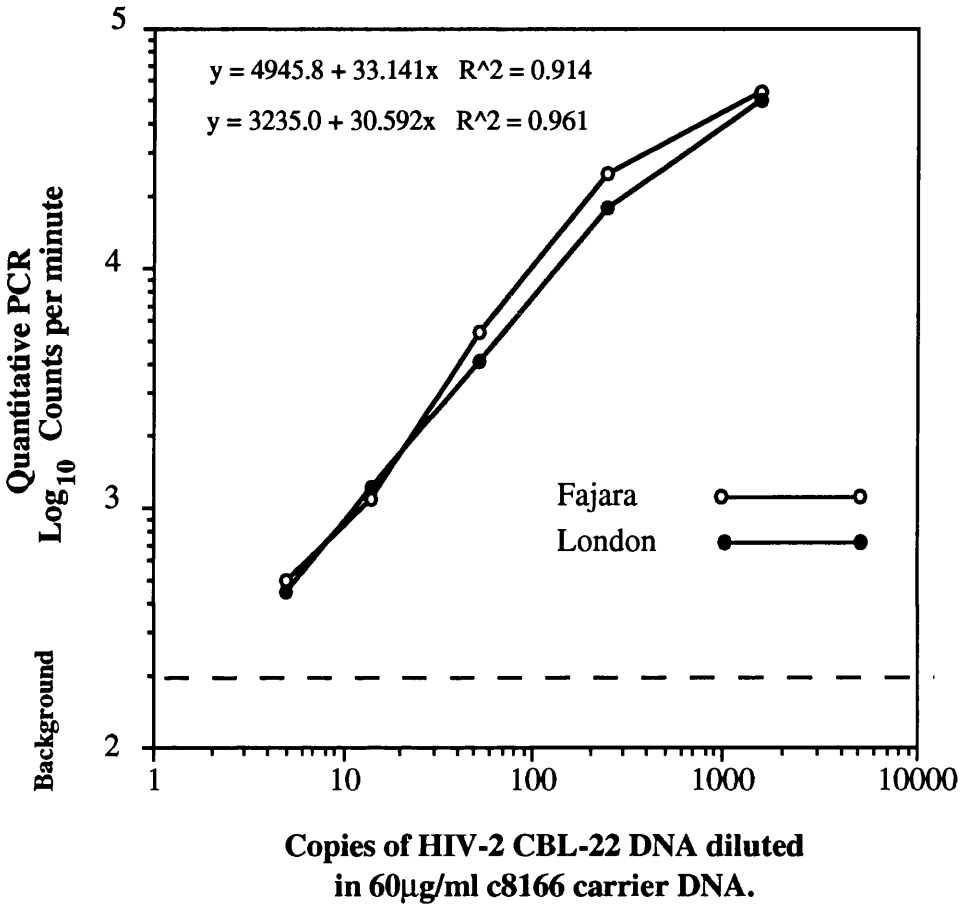


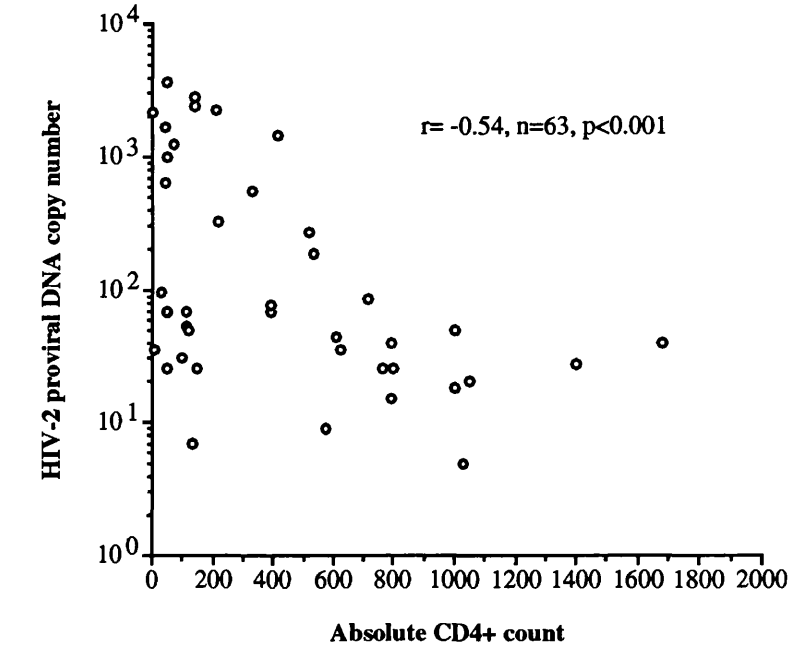
Figure 5.10. Regression curves for quantitative assays performed in London and Fajara.

Incorporation assays of this kind are based on linear regression whereby qPCR measurements are only valid if the external controls used provide data before the plateau phase of amplification is reached and the assay is in the linear phase. Linear regression was performed on assays performed in London and Fajara and are compared in Figure 5.10. The high regression values obtained on both is also indicative of reproducibility between the two sites. Comparison of duplicate readings of the same sample amplified in parallel indicated that most values were within 25% of each other as shown in Table 5, Appendix 2. Therefore the results of an additional 23 samples which were were also initially quantified by the incorporation assay tested in Fajara were added to the HIV-2 proviral load measurements of the 40 tested in London and the results from all 63 adult patients were analysed together.

v. Correlation between proviral load, CD4 count and clinical status.

The relationship between proviral DNA levels and the immunological status (CD4 count) of HIV-2-infected individuals was investigated. An excellent correlation between qPCR and limiting dilution had been previously demonstrated and so the qPCR data only were used in subsequent analyses, which included an additional 23 samples tested in Fajara, representing a total of 63 Gambian individuals. HIV-2 copies per 10^5 PBMC's were subsequently adjusted to account for the proportion of CD4+ cells remaining in the peripheral circulation (%CD4) and the clinical status of the 63 individuals was related to viral load measurements.

The data were initially compared by two similar analyses to determine the general reationship between the CD4 count and proviral load. The actual copy number per 10^5 PBMC's was compared with the absolute CD4 count and then the same analysis performed, adjusting for the %CD4 count. The general trend observed with each of these analyses was an inverse relationship between the proviral DNA load and CD4 count (Figure 5.11.). Comparisons made using Spearman rank correlation analysis demonstrate a strong inverse correlation between these two parameters irrespective of whether the actual copy number as determined by the qPCR assay directly are used, or where copies are expressed according to the proportion of CD4+ cells remaining in the peripheral circulation.



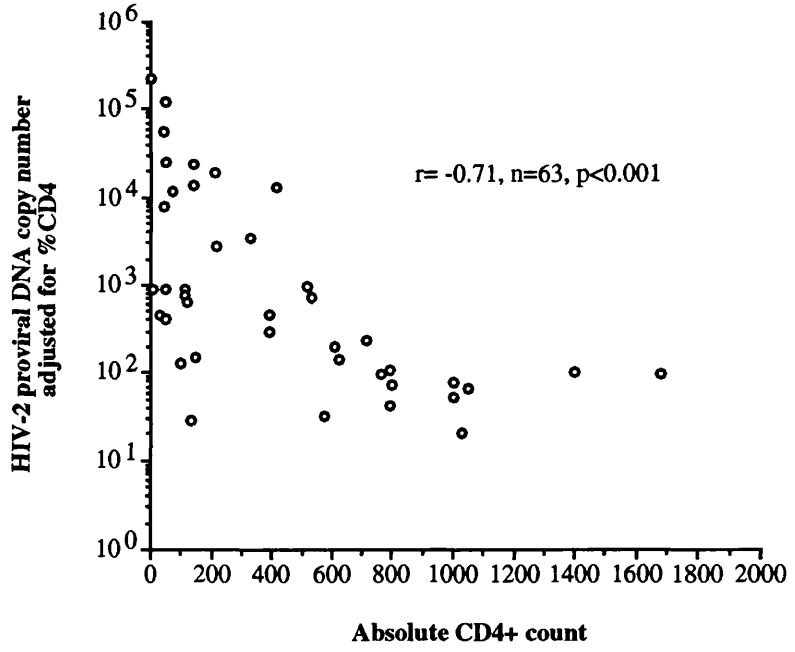


Figure 5.11. Comparative analysis of proviral load measurements with absolute CD4 counts in 63 HIV-2-infected individuals. Comparison of viral load data expressed both as copies per 10^5 PBMCs (upper) and 10^5 CD4+ cells (lower) with absolute CD4 counts using LTR primers. Spearman correlation analyses are indicated in each case.

In patients where the CD4 count is very low (<200 cells/mm^3), a substantial proportion of infected cells available for amplification are no longer present as a consequence of HIV-2-related disease and therefore the copy number obtained from these individuals may be artificially low as a consequence of lymphocyte depletion. By taking into account the %CD4 values for these patients, the proviral DNA levels are adjusted such that a more accurate comparison with patients with lower viral loads and higher CD4 counts could be made. Therefore, the level of HIV-2 proviral load determined by qPCR in 10^5 PBMC's showed an inverse correlation with CD4+ cell number ($r= -0.54$, $n=63$, $p<0.001$,) which when the level of integrated provirus was directly related to the differing levels of CD4+ cells in the peripheral circulation of individual patients increased to show a more significant inverse correlation ($r= -71$, $n=63$, $p<0.001$). The overall relationship between CD4 count and viral load is shown in Figure 5.12. including the two seropositive individuals who remained PCR-negative and in whom the absolute numbers of CD4+ cells was high, implying a very low level of virus-infected cells in these two individuals.

The distribution of HIV-2 genome levels in the 63 adult patients was also compared by stratifying the CD4+ count into 3 groups or bands according to the revised CDC classification (Table 5.3.). Although overlapping, the levels are clearly different. The mean $\log_{10}$ proviral load, expressed as copies of proviral DNA per 10^5 CD4+ cells for the high (>500), middle (200-499) and low (<200) band of CD4+ cells was 1.88 ± 0.43, 2.84 ± 0.93 and 3.4 ± 1.05 respectively ($p<0.001$, Kruskall Wallis analysis of variance). On average, patients whose CD4+ count was less than 200 cells/mm^3 had a proviral load which was 33 times higher than in patients whose CD4+ counts were 500 or above.

The measurement of CD4 counts represents one means of stratifying patients. Since both the absolute numbers and percentage of CD4 cells were measured, the 1993 revised classification of grouping AIDS patients was used rather than alternative diagnoses of AIDS such as the Karnofsky score or the Bangui clinical case definitions both of which were intended for use in an African setting. The overall relationship between viral load, CD4 count and the CDC clinical categories was also investigated and is summarised in Table 5.4.

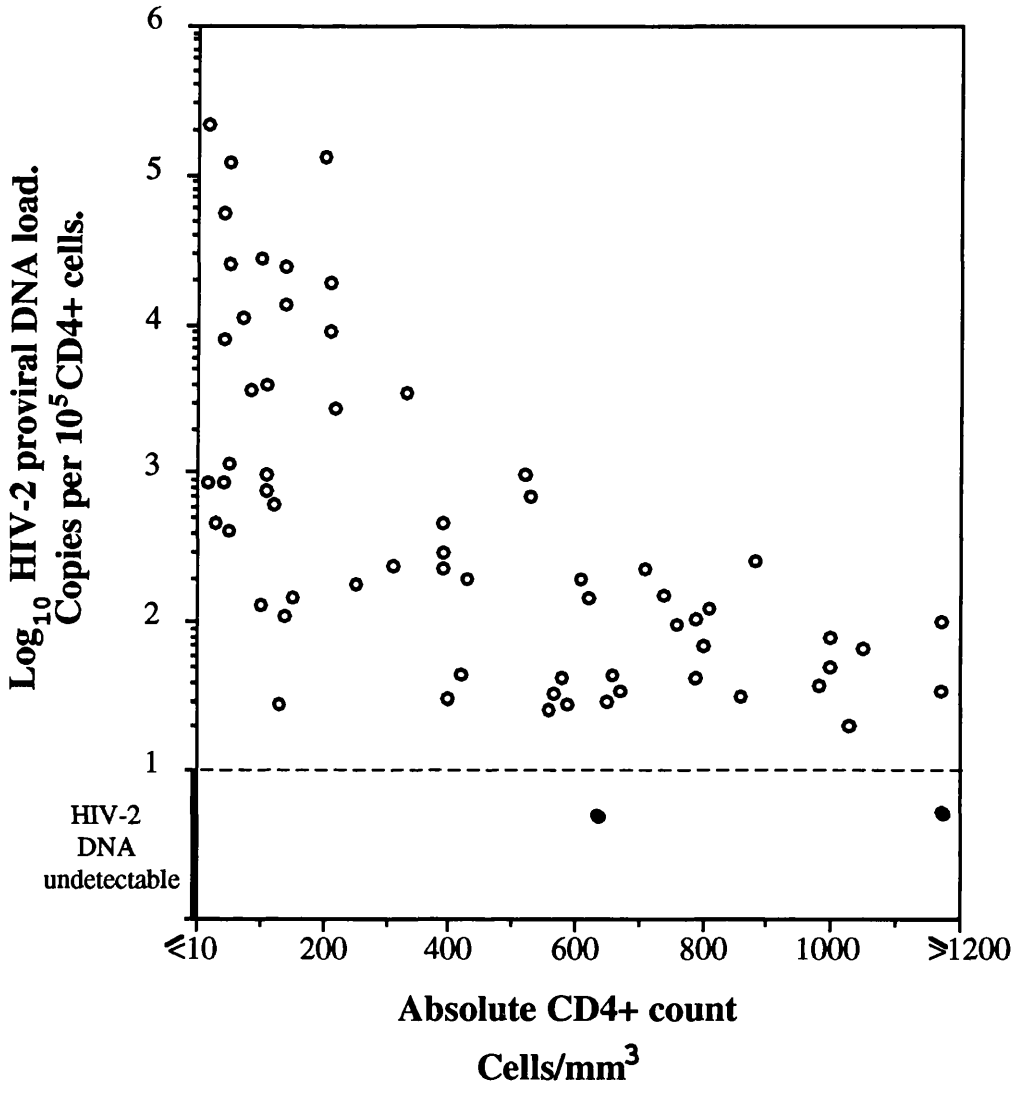


Figure 5.12. Inverse relationship between virus load and absolute CD4 count in 63 adult HIV-2 infected individuals. The two solid datum points indicate individuals who were HIV-2 seropositive but remained PCR negative with both LTR and *pol* primer sequences.

Table 5.3. Relationship between proviral load and CD4 grouping.

CD4 group	n	Mean (SD) Log_{10} qPCR
CD4 <200	23	3.40 (1.05)
CD4 200-499	13	2.84 (0.93)
CD4 >500	27	1.88 (0.43)

Kruskall-Wallis analysis of variance between CD4 groups, $p<0.001$.

Table 5.4. Relationship between proviral load, CD4 count and clinical status.

Clinical status	n	Mean (SD) Log_{10} total CD4 count	Mean (SD) Log_{10} virus load
CDC A	17	2.67 (0.40)	2.27 (1.02)
CDC B	20	2.64 (0.37)	2.31 (0.76)
CDC C	26	2.04 (0.63)	3.11 (1.11)

Kruskall-Wallis analysis of variance between CD4 strata for absolute CD4 count ($p<0.001$), and for quantitative PCR ($p<0.02$). Quantitative PCR values are expressed according to the percentage of CD4 cells remaining in the peripheral circulation.

In general, patients in CDC category C (AIDS) had seven times the viral load of patients in CDC category A who were asymptomatic or had persistent generalised lymphadenopathy, though the differences in viral load between the clinical categories were not as marked as those between the categories for CD4 counts. Although the relationship between clinical status and virus load was not as strong as that observed between virus load and CD4 count, it was evident that patients who were overtly symptomatic had proportionately higher levels of circulating virus than individuals who had mild or no symptoms of HIV-2-related disease. This may be a reflection of the time incurred between the appearance of HIV-2-related clinical disease compared with the more directly obtainable measurements of CD4 counts and viral load. Despite this, raised virus load levels were associated with a higher frequency of clinical symptoms compared with patients with lower virus loads who were largely asymptomatic.

5.c. Discussion.

5.c.1. PCR efficiency and primer design.

Three regions of the HIV-2 genome were targetted for nested PCR amplification, the U3/R region of the LTR and the *pol* (integrase) and *vpx* genes. When embarking on this work, the number of reports in the literature describing the detection of HIV-2 proviral DNA in peripheral blood were few. However, these served as useful guides to the design of nested PCR primers for each region and although some of the sequences used are similar to some published sequences, they are not identical and the combination of four individual primers in fully nested PCR assays are different to any previously described.

The efficiency of any given PCR reaction is determined by numerous factors, one of the more important ones being the compatibility of the primers used in the reaction for the DNA target sequence. The HIV-2 LTR nested primer set was designed by modifying the SK89 and SK90 primers originally described for investigations of mixed HIV-1 and HIV-2 infections in PBMC DNA extracted from individuals from the Ivory Coast (Rayfield *et al*, 1988). Corresponding to functionally conserved regions within the U3/R region of the HIV-2 LTR, the location of these four primers is shown in Figure 5.13. indicating the relatively high degree of conservation compared with other HIV-2 and SIV strains. In this region, three Sp1 enhancer sequences are located, relating to DNA binding sequences for cellular transcription factors. The 5' outer primer ends in a GGG sequence which is conserved for HIV-2 and SIV strains. Of the four primers, however, this displays the least sequence conservation, particularly with the SIV strains, although there are only minor mis-matches with most HIV-2 strains. The next three bases in the overall consensus sequence (AGG) are also absolutely conserved and therefore for the initiation of strand elongation and for binding of the inner sense primer, 100% conservation was achieved. Similarly at the 3' end of the inner sense primer which ends CCCT, a natural T is incorporated the next base of which is also a T in most HIV-2 strains to maximise primer extension (Kwok *et al*, 1990).

The 3' inner and 3' outer primers are in an even more conserved region of the genome corresponding to part of the TAR (*tat* acceptor region) core in the repeated (R) part of the LTR whereby only 2 base changes in a possible 760 across all HIV-2 and SIV strains

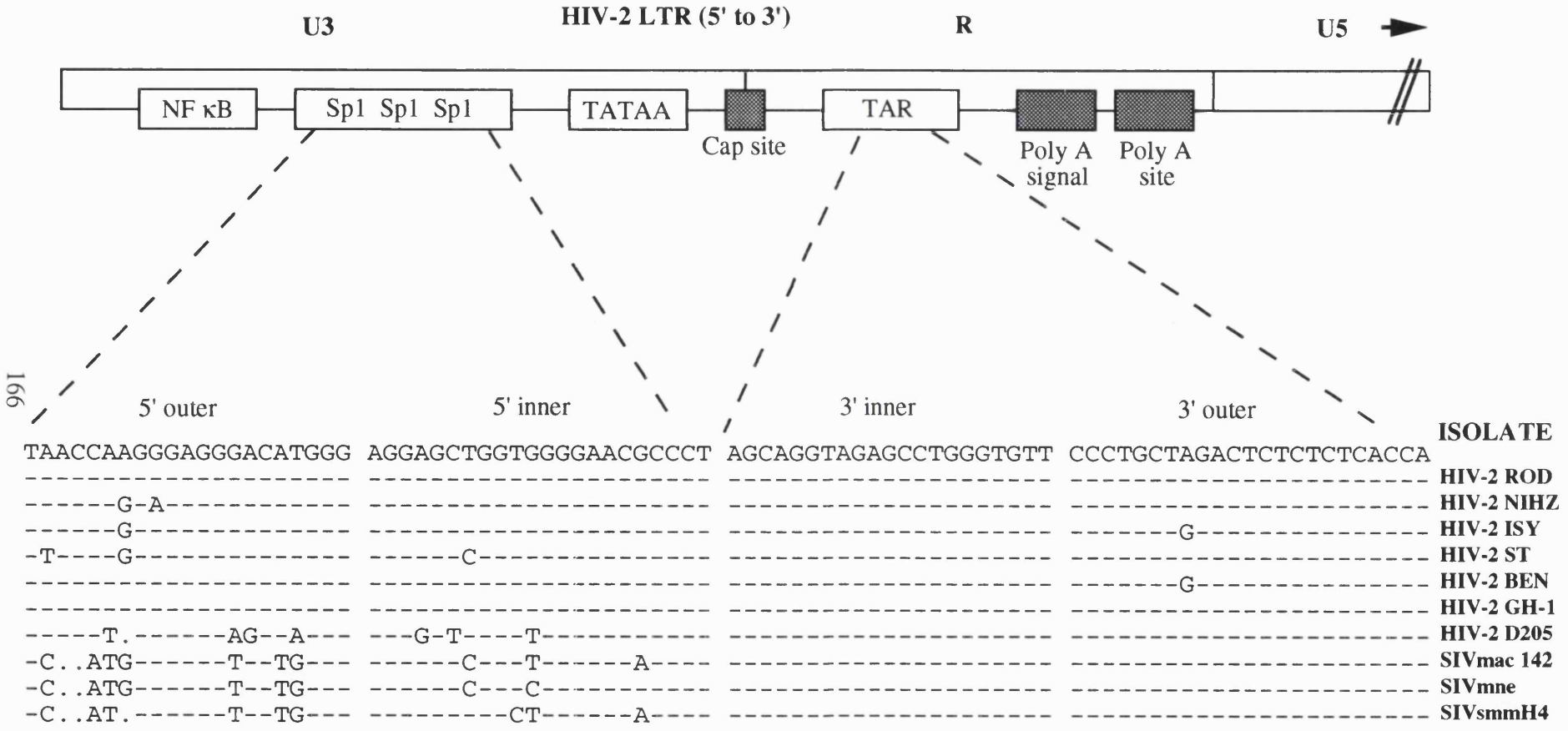


Figure 5.13. Sequence conservation of diagnostic LTR primer sequences across a range of HIV-2 and SIV isolates. The primers were designed principally using the consensus of subtype A strains although the sequences of HIV-2 D205 (subtype B) and several SIV strains are shown for comparison. Primers were designed to cover the Sp1 sites and the TAR region of the HIV-2 genome.

(19 strains in total in the 1993 database) are evident. In HIV-2_{ISY} (a Gambian strain) and HIV-2_{BEN} there is an A to G mutation in the outer antisense primer although is likely to have minimal effects on primer binding. For diagnostic purposes, the LTR represents a good target for primer binding since sequences are highly conserved, in particular the TAR region, due to functional conservation in HIV-2 gene regulation. A more detailed analysis of the specific elements in the HIV-2 U3 LTR are described in Chapter 6.

In preliminary studies, the LTR primers were compared with other primer combinations in a conserved region at the 3' end of *pol*, using the universal primer system (UNIPOL) which amplified a 336bp region within the integrase gene (Miura *et al*, 1990). Amplification of HIV-1, HIV-2, SIVmnd and SIVagm viruses and subsequent southern blot analysis of the amplified fragment with the appropriate DNA probe allowed differentiation of the PCR products according to the species of origin. Miura *et al*, (1990) also demonstrated that HIV-2 strains such as the strain GH-2 were also amplified with the UNIPOL primers, and DNA sequence analysis of the internal region indicated the presence of a divergent genotype (HIV-2 subtype B viruses).

The UNIPOL primers were first applied in a hemi-nested PCR with the initial objective of amplifying both HIV-1 and HIV-2, including both putative HIV-2 subtypes from clinical material. Although it may be possible to demonstrate the presence of DNA from the four major phylogenetic groups by using a species-specific probe following amplification with conserved primer sequences using relatively high concentrations of target DNA from each species, this primer configuration was found to have severe limitations when applied to clinical specimens. The UNIPOL primers were therefore re-designed to yield a fully nested primer set in *pol*, required to achieve the necessary specificity with clinical samples and to maximise detection of HIV-2/SIVsm viruses. Where possible, native sequences were employed although neutral inosine residues were introduced at regions of known hetereogeneity despite the low level of overall genetic variation in this region. Consecutive regions of conservation were utilised in primer design, as with the LTR primer set. The outer and inner sense primer sequences overlap by 5 bases (AGTGG) which is 100% conserved across all HIV-2/SIV sequences on the database and the inner sense primer which ends GCAGA is similarly conserved. The

antisense sequences are also located in two regions of low variation. This produced a set of primers which would amplify with the greatest efficiency HIV-2/SIVsm strains but the sequences were sufficiently different from the UNIPOL set, such that HIV-1 strains were not detected as demonstrated by their lack of reactivity with high copies of HIV-1 DNA (Figure 5.3.). The hemi-nested primers may be useful in any future studies for detection of recombinant viruses since sequencing of the internal region would identify a genetic variant, although the spurious satellite bands generated with these primers may be unrelated to retroviral infection.

Two nested PCR primer sets in putatively highly conserved regions of the genome (LTR and *pol*) were therefore derived and used to determine the presence of HIV-2 proviral DNA in a group of HIV-2 seropositive individuals in The Gambia. LTR primers were capable of single molecule detection with both HIV-2 and SIVmac DNA samples. Analysis of the database also indicated that these primers would detect HIV-$2_{ALT(D205)}$. The few published reports of HIV-2 proviral DNA detection in PBMC's describe the inability to amplify HIV-2 DNA from approximately 15% of HIV-2 seropositive individuals. Notably, Grankvist *et al*, (1992) used nested PCR primers in LTR, *gag*, *pol* and *env* regions and were able to amplify DNA from 31/37 (84%) individuals with at least one of the nested primer sets and 23 of the 37 samples (62%) were positive with at least three regions. The results obtained in the present study compare favourably with those obtained by Grankvist and colleagues since it was possible to obtain PCR signals using DNA from 84/86 (97%) of seropositive individuals with the LTR primer set and 39/41 (95%) with the modified *pol* nested primer set. The numbers of patients and samples tested was also higher in the present study and the approach adopted also different whereby two sets of nested primers designed specifically in putatively highly conserved regions of the genome were applied.

A number of explanations may account for these apparent differences. The samples tested in the current study were all either from Gambian individuals or from individuals present in The Gambia at the time. At least two of the samples not detected by Grankvist *et al*, (1992) were from individuals from the Ivory Coast. Serological mis-classification is a possibility given that antibody assays exhibit a higher level of cross-reactivity on

samples from the Ivory Coast than in other parts of West Africa. It is also possible that a higher proportion of subtype B viruses are present in the Ivory Coast compared with western West Africa. Primers designed using a database consisting principally of subtype A viruses may be inadequate for the design of effective primers for detection of subtype B viruses. The general observation may be made that a higher proportion of PCR 'misses' are evident with samples from the Ivory Coast included in studies where individuals are from different nationalities. The relative proportions of HIV-2 subtypes in different geographical regions of West Africa remains unclear. Low viral load, possibly also as a reflection of virus subtype, may also account for PCR misses and remains the most likely explanation.

4.c.2. Dual HIV-1 and HIV-2 infections.

The rise in prevalence of HIV-1 in West Africa, with the accompanying increase in the number of AIDS cases and HIV-disease, into a region endemic for HIV-2 has led to the need for accurate identification of HIV-1, HIV-2 and dually-infected individuals. The phenomenon of dual seropositivity and therefore the implication of dual infection with HIV-1 and HIV-2 has been particularly common in the Ivory Coast although this is now happening in other parts of West Africa, including The Gambia.

Most of the studies concerning dual infections have been performed on samples from the Ivory Coast where PCR assays have been applied to samples obtained from individuals serologically dually reactive to determine the true prevalence of HIV-1, HIV-2 and mixed infections (Rayfield *et al*, 1988; Peeters *et al*, 1992; Leonard *et al*, 1993; Peeters *et al*, 1994a). In one case, simultaneous isolation of HIV-1 and HIV-2 from a patient with AIDS was obtained which is perhaps the strongest evidence for dual HIV-1 and HIV-2 infection (Evans *et al*, 1988b). In The Gambia, dual reactions in serological assays have occurred less frequently, although dual infections were demonstrated using HIV-1 and HIV-2 nested PCR assays. Diagnosis of HIV-1, HIV-2 or dual infection by a combination of serological and PCR assays remains a difficult issue, however. The advantage of using HIV-1 *pol* and HIV-2 LTR primers on dual serologically reactive samples was that both primer sets had been evaluated on significant numbers of patient specimens from HIV-1-infected individuals (Kaye *et al*, 1991) and HIV-2-infected

individuals. The choice of primers and strategy employed for their application is therefore important.

The *vpu* and *vpx* primers were originally intended for the identification of dual infections by the detection of type-specific HIV-1 and HIV-2 genes respectively. However, in practice their role was limited. The *vpx* primers also failed to detect SIVmac DNA, although the detection of independent *vpu* and *vpx* gene sequences in the same patient provided firm evidence of co-infection with HIV-1 and HIV-2 in this individual. African strains have also been demonstrated to pose problems for PCR amplification (Candotti *et al* 1991; Grankvist *et al*, 1991). This was demonstrated using the *vpu* primer set which failed to amplify with strains U455a and Z84, both of which of African origin.

The LTR primers for HIV-2 detection and *pol* primers for HIV-1 detection provided the strongest predictive values for genome identification. Peeters *et al*, (1992) using samples from the Ivory Coast conclude that approximately one third of all dually reactive samples by serological assays were PCR positive for HIV-1 and HIV-2, thus representing genuine dual infections. This was also found to be the case in a study of mixed HIV-1 and HIV-2 infections in Brazil where PCR assays for HIV-1 and HIV-2 were used following an immunoassay based on viral lysate antigens and type-specific peptide assays (Pieniazek *et al*, 1991). Amplification of HIV-1 and HIV-2 protease genes yielding a 300bp fragment, which following restriction endonuclease cleavage by *Alu*I for HIV-1 specificity and *Hinf*I for HIV-2 specificity, allowed speciation of the infecting HIV-type. Restriction endonuclease cleavage patterns with a number of different enzymes in conjunction with sequence analysis of the amplified product excluded the possibility of a recombinant HIV-1/HIV-2 variant. Restriction digest patterns have also been used for the speciation of HTLV-1 and HTLV-2 infections where amplification of *tax/rex* of HTLV-1 or HTLV-2 is followed by digestion with *Taq* 1 and *Sau* 3a respectively for speciation (Tuke *et al*, 1992). The higher degree of conservation within the HTLV group allows such an approach which is more difficult to achieve with HIV in view of the sequence variation both within and between the HIV-1 and HIV-2/SIVsm groups.

Differences in methodology may also play a contributory role in identification of dual infections. Peeters *et al*, (1994a) were unable to amplify HIV-2 proviral DNA sequences from primary uncultured material of seropositive individuals. By performing co-culture of lymphocytes for up to six weeks, followed by PCR with HIV-1 and HIV-2 primers the success rate of amplification was increased suggesting that provirus was originally present but below the levels of detectability, which upon stimulation was brought into the range available for PCR amplification. The overall sensitivity of the HIV-2 primers was ultimately demonstrated to be 95.6% which is similar to that described in this thesis. It was also demonstrated that 48% of sera from dual seropositive individuals were capable of neutralising simultaneously both HIV-1 and HIV-2 prototypic isolates. This compares with 24% of sera containing anti-HIV-1 only and 17% containing anti-HIV-2 only, which further suggests that differences in the biology of HIV-1 and HIV-2 infections compounds speciation. The phenomenon of low virus load in HIV-2-infected individuals may therefore compromise the ability to diagnose dually-infected persons either by direct PCR amplification from PBMC DNA or in conjunction with co-culture techniques.

Most of the reports described where HIV-2 PCR assays have failed to detect provirus in PBMC DNA have therefore alluded to the possibility that HIV-2-infected individuals may be infected with a relatively low level of virus, thereby falling below the threshold of detectability. Often these samples were obtained from asymptomatic individuals with relatively high CD4 counts. In our study the two individuals in whom HIV-2 sequences could not be detected by either LTR or *pol* primers, both had CD4 counts greater than 500cells/mm^3. The relationship between proviral DNA load and the immunological status in HIV-2-infected individuals in The Gambia was therefore formally investigated.

4.c.3. Viral load studies.

A quantitative radiometric assay was developed for the investigation of HIV-2 proviral DNA load in HIV-2 infected individuals, intended for use both at the MRC laboratories in The Gambia and within the Department of Virology, UCLMS. The assay is conceptually relatively simple whereby the incorporation of a revealing agent, in this case [^{35}S] labelled dATP, into the amplified product of a secondary amplification reaction, is directly proportional to the input of specific HIV-2 DNA. The assay relies upon both the

increased specificity and sensitivity of a secondary PCR, the key to which is the consistent and reliable amplification of HIV-2 target sequences by the LTR primers. The increased sensitivity using *Pfu* DNA polymerase was also important not only because a larger dynamic range was attainable, but also in view of the potential for HIV-2 DNA to be integrated at low levels within HIV-2-infected individuals.

When establishing a new assay of any kind it is important to have an independent means of validation. Therefore the quantitative data obtained with the qPCR assay were compared with an estimation of copy number by end-point limiting dilution and calculated according to the Poisson formula. The high degree of correlation between the two methods served to validate the qPCR assay and to confirm the wide range of HIV-2 proviral DNA copies in the 40 HIV-2-infected individuals compared by both methods. The use of limit dilution is restrictive, however, and inappropriate for handling large numbers of patient specimens when compared to the qPCR assay. Comparison of the two methods indicated a good agreement either when the data were expressed per 10^5 PBMC's or per 10^5 CD4+ cells. This allowed not only an independent validation of the qPCR assay but also indirectly addressed the possibility of inhibitors within DNA preparations which were relatively crude having undergone no additional purification. However, no significant levels of inhibition were observed which if had been present would have become apparent from limit dilution studies and discrepansies in the two values observed. In fact, a wide range of proviral levels were identified, irrespective of the method used and the overall correlation was high. If inhibitors were present their effects appear to have been marginal which is also a reflection of the potential of nested PCR to amplify specific target DNA sequences from a background of cellular DNA and protein.

The qPCR assay described uses an external strandard control. Some investigators also include a control for human DNA by amplifying endogenous marker sequences such as HLA or β-globin (Ferre *et al*, 1993). This not only confirms the presence of human DNA in the sample but gives an independent value in terms of the efficiency of the PCR reaction on that particular specimen. Alternatively, an internal control for the expected sequence is co-amplified with the sample to provide, in theory, a more specific assay.

This competitive approach has been employed to measure HIV-1 RNA levels in patients at all stages of HIV-1 disease (Piatek *et al*, 1993). Competitive PCR assays, however, are expensive requiring multiple replicates and do not make for ease of handling relatively large sample numbers.

Using the quantitative PCR assay developed it was demonstrated that HIV-2 proviral load is inversely related to the CD4+ lymphocyte count in the peripheral blood of HIV-2-infected individuals. The CD4 molecule is most likely to be the principal target for infection in the peripheral blood (Schnittman *et al*, 1989; McElrath *et al*, 1989). To take into account the different levels of CD4+ cells at different stages of disease, HIV-2 proviral load were expressed per 10^5 CD4+ lymphocytes. Some individuals had very low levels of integrated provirus including one patient with 20 copies/10^5 CD4+ lymphocytes detectable by quantitative LTR PCR which was not detected by the *pol* primers. Proviral load was demonstrated to be exclusively low in patients with CD4 counts >500 cells/mm^3, and overall a significant inverse relationship between HIV-2 proviral load and CD4+ lymphopaenia was clearly demonstrated. The range of virus load in patients with a CD4 count of less than 500 cells/mm^3 is similar to the published data of HIV-1 load in patients infected with HIV-1 (Yerly *et al*, 1992; Jurrians *et al*, 1992a; Bieniasz *et al*, 1993). In studies of HIV-1 proviral load, the numbers of CD4+ cells have also been adjusted and comparison of proviral DNA levels in patients at different stages of disease, reflected principally in the proportion of CD4+ cells remaining in the peripheral circulation, also exhibited a similarly wide range of virus load (Ferre *et al*, 1992; Wood *et al*, 1993). Levels of provirus ranged from 1 in 1000 to 1 in 10,000 CD4+ infected cells in asymptomatic individuals to as high as 1 in 10 CD4+ cells in advanced stages of disease. An increasing proviral load is therefore strongly associated with disease progression and related to severe immunodeficiency for HIV-1 (Schnittman *et al*, 1990; Ferre *et al*, 1992). This would also appear to be the case for HIV-2 although the time over which this occurs may be longer.

The circulating viral load in HIV-2-infected individuals has also been assessed using co-culture techniques where the levels of both cellular and plasma virus have been measured (Simon *et al*, 1993). The study by Simon and colleagues was the first formal published

report analysing viral load in HIV-2 infection. Forty adult patients (17 men and 23 women) were studied attending hospitals in France who originated from a wide range of West African countries. Quantitative cell and qualitative plasma viraemia assays for virus culture were employed as well as qualitative PCR measurements employing the nested PCR previously described by Grankvist *et al*, (1992). The prevalence of HIV-2 DNA detectable by PCR was similar to that previously described (Grankvist *et al*, 1992) where HIV-2 DNA was detectable in approximately 85% of seropositive patients. In the study of Simon and colleagues, four patients remained negative by HIV-2 nested PCR, three of whom were from the Ivory Coast and all of which had a high CD4+ count. Where both cell and plasma viraemia levels were measured there was also an inverse correlation with the rate of HIV-2 isolation and CD4 count. However, although HIV-2 load in patients with a CD4 count <200 was similar to data obtained with HIV-1, the isolation rate from plasma was found to be significantly lower than for HIV-1. This trend was repeated when higher CD4 counts were compared. Both the rate of isolation from plasma and the quantitative measurement of cellular load was significantly lower for HIV-2 where CD4 counts were between 200-500 and >500 cells/mm^3. The level of virus in these patients was taken as a reflection in the ability to detect HIV-2 genome by PCR. While the measurement of infectious virus may be important when investigating virus load in relation to HIV-disease and for making comparisons of HIV-1 and HIV-2, there are certain limitations when dealing with culture-based protocols and methodologies. However, the similarity between the levels of virus in HIV-1 and HIV-2-infected patients with a CD4 count of <200 is particularly striking measured either by qPCR or culture techniques, akin to clinical similarities between HIV-1 and HIV-2 in end-stage disease.

The application of sensitive PCR assays in asymptomatic patients with a high CD4 count is likely to offer certain advantages over culture-based techniques for quantification of HIV-2. In the HIV-2 DNA study described in this thesis it was possible to detect provirus at levels as low as 20 copies per 10^5 CD4+ lymphocytes, equivalent to 8 copies per 10^5 PBMC's. Although this is close to the lower limit of detectability of the qPCR assay with an input of 10^5 PBMC's, it was possible to obtain quantitative measurements by LTR qPCR in all but two patients both of which had high CD4 counts. It was also apparent that there were no patients in the study with high levels of virus load

accompanied by high CD4 counts. In HIV-1 infection, low viral load has been linked with a slower disease progression (Schechter *et al*, 1991) and it seems likely that this feature of infection may also be an important correlate in the longer incubation period and apparently lower infectivity of HIV-2. More studies of viral load in HIV-2-infected individuals are required to attain a clearer picture of the role of viral load particularly where HIV-2 has been present for a significant number of years.

Explanations for the differences between PCR and culture-based methodologies may be proposed, such as the detection of defective, non-infectious virus by PCR which would not yield viable virus in culture systems. HIV exists *in vivo* as a 'quasispecies' consisting of multiple variants of virus, particularly as integrated provirus which remains as a library of sequences within an infected individual (Meyerhans *et al*, 1989; Goodenow *et al*, 1989; Wain-Hobson, 1989). Quantitative PCR techniques based upon proviral DNA amplification cannot distinguish between defective provirus and infectious virus. However, in HIV-1 infection it has been demonstrated that by sequencing *env*, the proportion of defective virus was minimal (Simmonds *et al*, 1990). Low levels of HIV-1 provirus in CD4+ T cells have also been linked with a high proportion of replication-competent viral sequences (Brinchman *et al*, 1991). Nucleotide sequence analysis of HIV-2 strains from 12 HIV-2-infected individuals from Guinea Bissau, where the putative region of the HIV-2 V3 loop was analysed, revealed additional information of HIV-2 infection *in vivo* (Boeri *et al*, 1992). The reading frames containing the V3 loop sequences identified were open, although it is unclear whether these encoded for functional proteins due to the limited sequence data available.

Defective genomes in HIV-2-infected individuals have been identified by sequencing HIV-2-related lentiviruses closely related to SIV strains (Gao *et al*, 1992). Notably, it was not possible to culture the virus HIV-2_{F0784} for which such sequences were described but its identification relied upon amplification by nested PCR. HIV-2_{F0784} was subsequently identified to be phylogenetically indistinguisable from SIVsm sequences in the databank but was also found to have a high level of mutation of G for A in both *pol* and *env* genes. However, this strain may not be entirely representative of the majority of HIV-2 strains infecting humans most of which are unlikely to be as a result of recent

cross-species transmission events from simian hosts to man. The generation of defective proviruses may be one strategy employed by HIV to avoid recognition by the immune system and as a consequence may provide a selection advantage over proviruses producing replicating virus (Coffin, 1995). The relative proportion of defective genomes in HIV-2 infection remains to be investigated in detail.

It was shown, however, that a strong correlation between increased proviral DNA levels and CD4 depletion exists for HIV-2, although as with HIV-1 the measurement of cell-free virus in serum or plasma may give a better indication of replication-competent viral activity. There is, however, only a limited amount of information that can be gained from cross-sectional studies of the kind described here. Longitudinal studies investigating the temporal expression and viral dynamics in HIV-2 infection may provide further insight into the relative expression of HIV-2 infections *in vivo*, although these may be difficult to establish in view of the longer phase of asymptomatic infection in HIV-2-infected individuals.

Measurements of virus load, however, either proviral or virion loads, do not provide any indication as to the type of infecting strain. The proportion of different HIV-2 subtypes in this collection of samples was therefore investigated by PCR amplification and DNA sequencing of the U3 part of the 3' LTR. These studies are described in Chapter 6.

Chapter 6.

Genetic analysis of the HIV-2 LTR.

6.a. Introduction.

The repertoire of HIV-2 strains potentially available for infecting humans is considerable. At least three major groups of strains have been identified, designated subtype A (prototypic, HIV-2_{ROD}), subtype B (alternative, HIV-2_{D205}) and SIV-related strains (SIV/HIV-2_{F0784}). There may be other groups in due course, similar to the situation in HIV-1 infection, but these sub-divisions serve as a useful guide until refinement of strain genotyping has been performed. The bulk of the isolates characterised to date belong to subtype A, even those with differing biological properties such as HIV-2_{ST} (Kumar *et al*, 1990) and HIV-2_{ALI} (Taveira *et al*, 1994). However, since most prototypic isolates have been obtained from patients with AIDS and the SIV-like variants were identified in asymptomatic individuals by molecular rather than biological techniques, there may be sub-populations of HIV-2 strains which have a different disease association (Gao *et al*, 1992). Specimens on which proviral load data had been generated (Chapter 5), were investigated for the presence of SIV/HIV-2_{F0784} viruses.

Using nested HIV-2 LTR primers some patients were demonstrated to have high CD4+ counts and low levels of integrated provirus. It is likely, but untested, that these viruses would have been difficult to culture. In order to determine whether these could be more related to SIV/HIV-2_{F0784}, a simple PCR-based genotype assay was established by amplification of the *nef* gene and the U3 part of the LTR, which included the 40-44bp deletion present in SIV strains and HIV-2_{F0784} (Gao *et al*, 1992). The presence of this deletion was used to screen samples, on a population basis, to differentiate prototypic HIV-2 strains from SIV-(like) strains. The U3 part of the HIV-2 LTR which encodes sequence-specific transcription factor binding sites, was further investigated for differences in this region within the HIV-2/SIV group. Clinical material was used for PCR and direct sequence analysis, and differences in U3 sequences within HIV-2 strains *in vivo* compared. Selective amplification of viruses based upon their U3 sequence was subsequently applied to the Gambian samples.

6.b. Results.

6.b.1. Amplification of the *nef*/U3 region of HIV-2/SIV.

Nested PCR primers were designed for amplification of the *nef*/U3 region of HIV-2 and closely related SIV-like viruses. The 'diagnostic' LTR primer sequences had been demonstrated to have a high sensitivity (97%) for detection of HIV-2 provirus in seropositive individuals and were also shown to be proficient at detection of putative SIVmac/sm sequences. However, these primers amplify a consensus sequence which was selected for its high level of conservation within both the HIV-2 and SIVmac/sm subgroups. Therefore, to allow the possibility of independently identifying SIV-related variants, a region extending from that covered by the 'diagnostic' LTR primer set into the *nef* gene adjacent to the 3' LTR was targetted for amplification. This region is shown in Figure 6.1.

The 3' antisense LTR primers located in the R region of the HIV-2 LTR were therefore maintained and combined in another nested PCR with an outer 5' (forward) sense primer at the start of *nef*, primer sequence originally described by Gao *et al*, (1992) and an internal primer designed from analysis of the 1992 Los Alamos Database (Table 2.1.). The two *nef* primers were theoretically capable of detecting HIV-2_{D205} and SIVmac located in regions of high sequence conservation. PCR amplicons of 692bp were generated for the first round for prototypic strains. Using the inner 5' primer amplifying from a conserved region within the *nef* gene, a 329bp second round PCR product was generated. As with other primer sets, the CBL.20-23 DNA preparations were used as well as HIV-2_{ROD} and SIVmac. Appropriate negative controls were included. The region amplified therefore encompassed the 40-44bp signature sequence of SIVmac/sm-related viruses which represents over one tenth of the region amplified and the entire U3 part of the 3' LTR. Amplified fragments were electrophoresed on high resolution agarose gels. All laboratory strains tested yielded amplification products with these primers (Figure 6.2.) where differences in amplicon size between the SIV and HIV-2 strains were clearly identifiable.

Unexpectedly, CBL-20 exhibited an intermediate band size (10-20 bp difference) between expected sizes for prototypic HIV-2 isolates and SIVmac. The smaller than

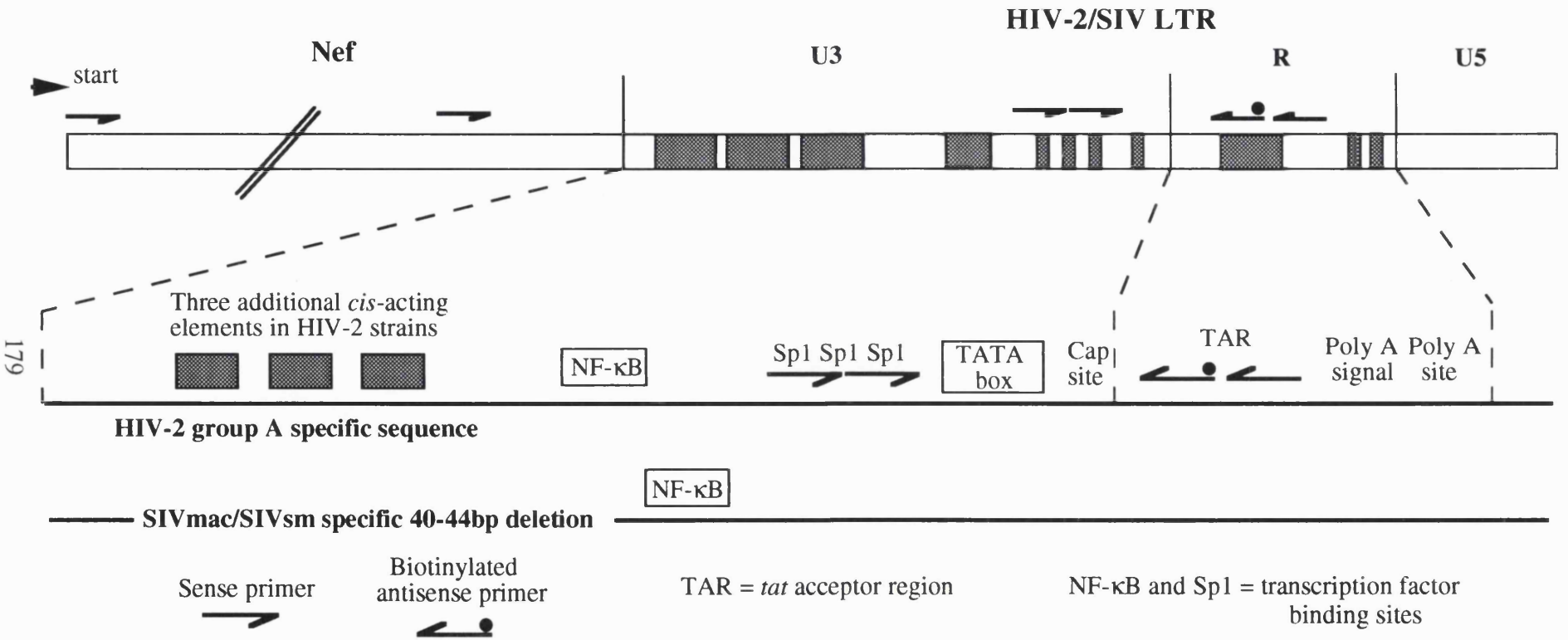


Figure. 6.1. Location of primers in the *nef* and U3/R region of the HIV-2 and SIV Long Terminal Repeat. The principal enhancer/promoter elements in the U3 LTR of HIV-2 group A strains and the transcription factor binding sites are indicated. The 40-44bp deletion in SIV strains is also shown and used to differentiate between putative SIV-like strains and prototypic-like HIV-2 strains on the basis of size difference of amplified products. The primers in the *nef* gene (sense) were used in primary and secondary amplifications with the antisense LTR primers.

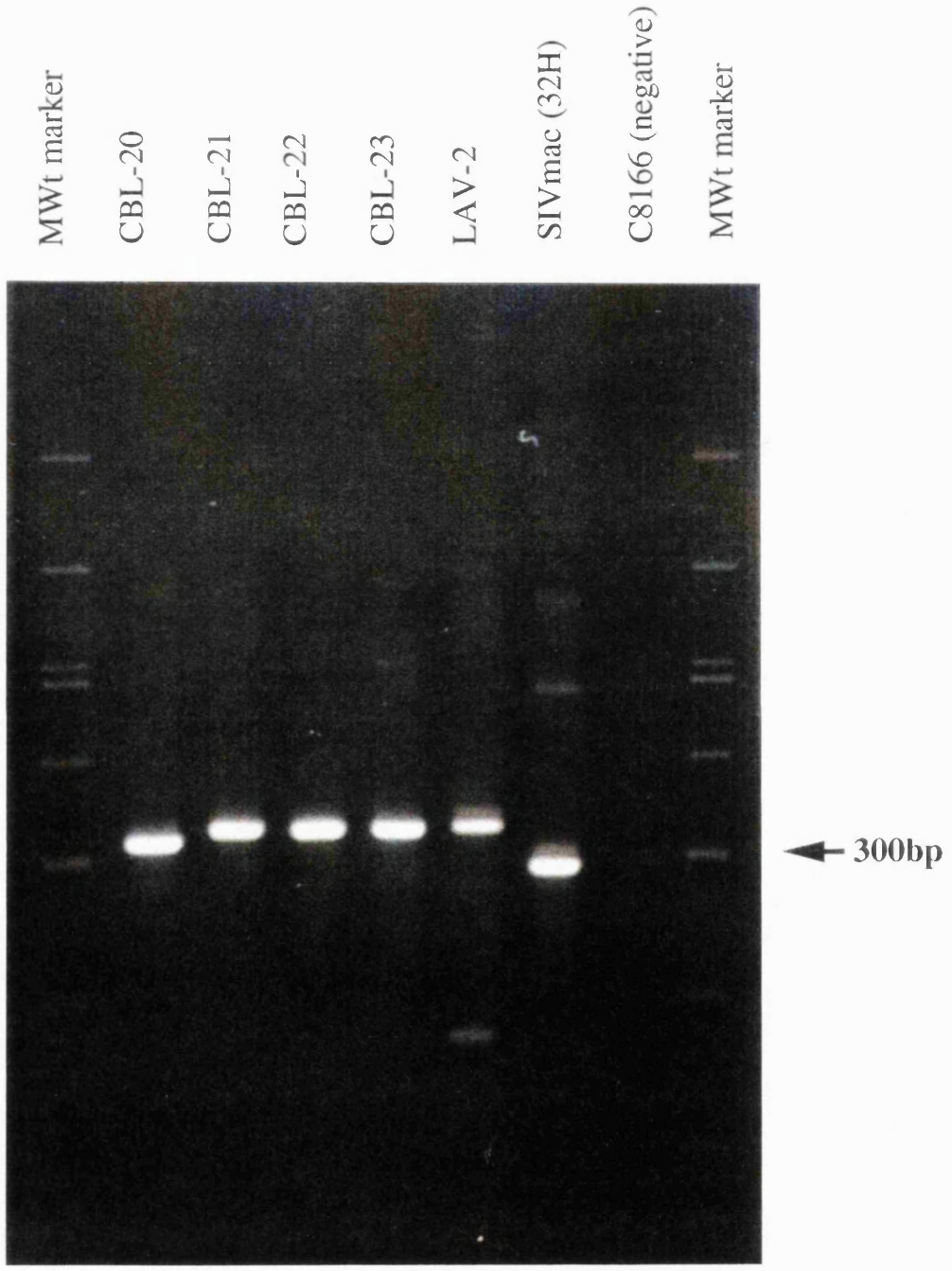


Figure 6.2. Differentiation of HIV-2 and SIV strains by electrophoretic mobility of LTR amplicons. The molecular weight ladder represents increments of 100bp.

expected size of CBL-20 was investigated by direct sequence analysis and the results described in 6.b.3. in conjunction with the RNA sequence data. The 3' inner antisense primer had been previously biotinylated for quantitative studies and the forward (sense) inner PCR primer was used as a sequencing primer. The first attempt to sequence this product resulted in a mixture of sequences and an uninterpretable autoradiograph. Single molecules were therefore generated by end-point dilution as described in previous sections to produce single PCR clones.

The *nef*/LTR primers, which reacted strongly with the other Gambian HIV-2 strains, HIV-2_{ROD} and SIVmac, were applied in the first instance to proviral DNA detection in the set of 40 PBMC-DNA samples for which proviral load data had been generated (Chapter 5). All 40 samples which were previously amplified with the diagnostic primer set were amplified with the *nef*/LTR primers. While the presence of a deletion in the CBL-20 isolate was an intriguing and unexpected result, it was not reproduced by analysis of the *in vivo* samples which all produced the predicted band size for prototypic strains. Three of these patient samples (P 975, P 1186, P1259) had very low circulating proviral load and were subsequently re-amplified such that single molecules were generated for each. This was performed using 20µl of DNA extract diluted in 400µl of PCR mix which after vortexing was distributed into 15 tubes and subjected to amplification. Bands were visible in 2/15, 2/15 and 7/15 samples for P975, P1186, and P1259 respectively, further demonstrating the single molecule detection capability with the *nef*/LTR primer set. The conclusion from these investigations were that no strains more similar to SIV than to HIV-2 were present in the samples analysed, on the basis of the 40-44bp signature sequence as a marker for SIV-(like) viruses.

6.b.2. HIV-2 RNA detection.

Since generation of single molecules was required to generate the CBL-20 DNA sequence, which was derived from cultured lymphocytic cell lines of CBL-20, the possibility existed that the deletion had been introduced during *in vitro* passage of this isolate. To ascertain if this was the case, an attempt was made to rescue cell-free viral RNA from stored serum samples of patient BM, from whom the original CBL-20 isolate had been derived. Similar samples from Gambian HIV-2-infected patients which had

been stored at -20°C since 1987 were also used. It should be noted, however, that the suitability of some these samples for the rescue of viral RNA was somewhat dubious even though they represented archival Gambian material. Other samples were also obtained from blood donors attending the Royal Victoria Hospital, Banjul, from whom relatively large volumes (20-30ml) had been obtained and stored aliquoted at -70°C. All were from different individuals to those previously tested for provirus detection. RNA was isolated using the RNAzol extraction protocol as described in Chapter 2.b.6 and cDNA generated by primer-directed reverse transcription using the outer antisense PCR primer. Nested PCR was subsequently performed with both diagnostic LTR and the *nef*/LTR primers. The results of these experiments are summarised in Table 6.1. comparing amplification efficiencies of both nested primer sets with samples stored either at -20°C or -70°C.

Of the two sample sets, only one sample stored at -20°C (BM) amplified with the diagnostic primer set, although the larger *nef*/LTR fragment was not amplified from this cDNA preparation. Therefore, it was not possible to determine whether the deletion observed with the CBL-20 isolate was present in virus *in vivo* in the patient. No other samples collected and stored under similar conditions signalled with either primer set. All samples stored at -20°C had, however, been frozen and thawed on several occasions for use in the preparation of conjugates in the original formulation of the competitive EIA. BM represented the homologous serum to the CBL-20 isolate and had remained largely untouched at -20°C in the intervening seven years, unlike the other samples.

Of the samples stored at -70°C and for which a larger volumes (10-20mls) of serum/plasma had been aliquoted, 5/7 (71%) signalled with the diagnostic primer set. Only 3/5 (60%) amplified with the *nef*/LTR primers. Since the total level of viral RNA in these patients was unknown, the number of cDNA molecules generated was calculated by the limiting end-point dilution method for each sample with the diagnostic LTR primer set. Two of these (BD1363 and BD1995) had slightly higher numbers of cDNA molecules available for amplification (9.5 and 22 molecules of cDNA respectively) than the remaining five all of which were close to the limits of detectability with the diagnostic LTR primer set (1-5 molecules of cDNA). This may, in part, explain why only samples

Storage -20°C			Storage -70°C		
Sample code	Diagnostic LTR	*nef*/LTR	Sample code	Diagnostic LTR	*nef*/LTR
Do. Nj.	-	-			
B-25	-	-	BD 1164	+	-
Sa.Se.	-	-	BD 1363	+	+
Mar.San.	-	-	BD 1688	+	-
Bo.Ma.	+	-	BD 1995	+	+
Fa.Se.	-	-	T 564	-	-
Mo.Bo.	-	-	T 1708	-	-
Ko.To.	-	-	T935/93	+	+
Maj.Sam.	-	-			

Table. 6.1. Diagnostic LTR and *nef*/LTR nested PCR primer reactivities with HIV-2 cDNA.

BD1363, BD1995 and T935/93 amplified with the extended *nef*/LTR primer set which requires approximately one kilobase of RNA transcript from the point of directed cDNA synthesis to mediate amplification. By comparison, the diagnostic primer set requires only 250-300bp of intact RNA for successful amplification. Hence the efficiency of amplification of the extended region may be a reflection of the frequency of long transcripts generated during reverse transcription. This would be the more favoured explanation for failure to amplify rather than primer mismatch due to sequence divergence, although this also remains a possibility. In these samples, therefore, which were obtained from blood donors the level of circulating cell-free virus in the peripheral circulation was low.

An additional and unexpected finding from amplification with the *nef*/LTR primer set was an insertion within the amplicon from patient T935/93. A comparison of the respective electrophoretic mobilities of the various amplified fragments was made on a single gel to demonstrate the heterogeneity of amplicon lengths with the *nef*/LTR primer combination (Figure 6.3.). The insertion in sample T935/93 and deletions in CBL-20 and SIV_{mac} are visible, compared with the predicted length of fragments amplified from either PBMC DNA or the other prototypic strains analysed. BD1363, BD1995 and T935/93 were sequenced in the U3 region and the sequence changes within the U3 LTR of these samples is described in 6.b.3. and compared with sequences derived from PBMC samples and laboratory isolates.

6.b.3. Sequence changes in the U3 LTR.

The region of the 3' HIV-2 LTR extending from the end of the *nef* open reading frame up to the NF-κB element was sequenced for laboratory isolates, PBMC DNA and viral RNA from a clinical source. Sequences could be obtained for most viruses up to and including the TATA box, although the U3 sequences immediately upstream of the TATA box were of most interest and attention was focussed on these. Direct sequence analysis of 'bulk' PCR product was performed to derive a consensus sequence except for CBL-20 and patient P1259 for which single molecules had been derived and three PCR clones generated to compare possible differences in HIV quasispecies. These, in fact, appear to be few with only minor changes found for each of the three PCR clones sequenced. The

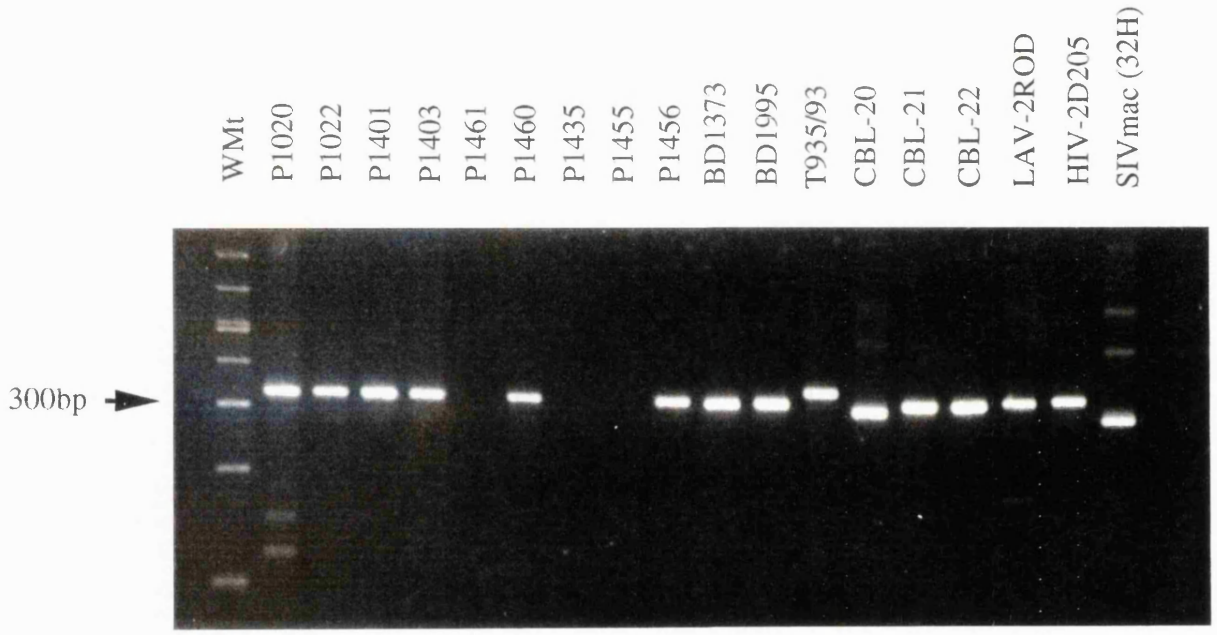


Figure 6.3. Heterogeneity of fragment length of LTR amplicons from a variety of sample preparations using type-common primers. The P series are PBMC DNA samples from either infected or uninfected patients. BD1373, BD1995 and T935/93 were amplified from cDNA (RNA source). HIV-2D205 is a subtype B strain.

derivation of single molecules for the CBL-20 isolate also allowed the sequence to be read with relative ease, the internal 5' PCR primer acting as the sequencing primer. The amplified product directly sequenced spans the end of the *nef* coding region into the 3' LTR up to the Sp-1 enhancer sequences which represent targets for diagnostic nested amplification. Consensus sequence was found to be sufficient in all other cases, particularly where RNA was the analyte providing clean and easily readable sequence.

Analysis of the CBL-20 sequence and comparison with CBL-21/22/23 strains indicated the length change was due to a 13bp deletion, which is upstream of the functionally conserved site NFκB enhancer element which is 100% conserved across all strains of HIV-2/SIV for which LTR sequences are available. For alignment comparisons, the Gambian strain HIV-2_{ISY} was used as a reference and all the sequences generated are compared in Figure 6.4. The 13bp deletion was only demonstrated in CBL-20 and not in the other Gambian strains (CBL.21-23). However the sequence in this region exhibits varying degrees of polymorphism (Figure 6.4.). The latter six bases (AAACAG) are conserved across all strains compared with the first seven bases which differ from each other and other HIV-2 strains. G for A mutations were the most common substitution although this mostly reflected differences between HIV-2_{ROD} and HIV-2_{ISY}.

The 13bp deletion in CBL-20 is intriguing and although not proven is possibly the result of continued passage in Jurkat and CEM cell lines. The intervening region between the PuB-2 and NFκB sites is more variable overall compared with the sequence-specific sites defining the other *cis*-acting elements most of which appear to be highly conserved *in vivo* within the subtype A group of viruses.

The repeated motif CTACTG*AAACAGCTGAGACTGCAG for sample T935/93 also aligns with the deleted sequence CTACTGAAAACAG in CBL-20, the first of which is perhaps the more significant finding since it has occured in cell-free virus amplified directly from the patient. The repeated sequence, although not an exact duplication is compared with other changes in this part of the LTR in Figure 6.4. Overall, this region is relatively highly conserved particularly the previously defined *cis*-acting sequences which act as transcription factor binding sites and are described in detail at the end of this

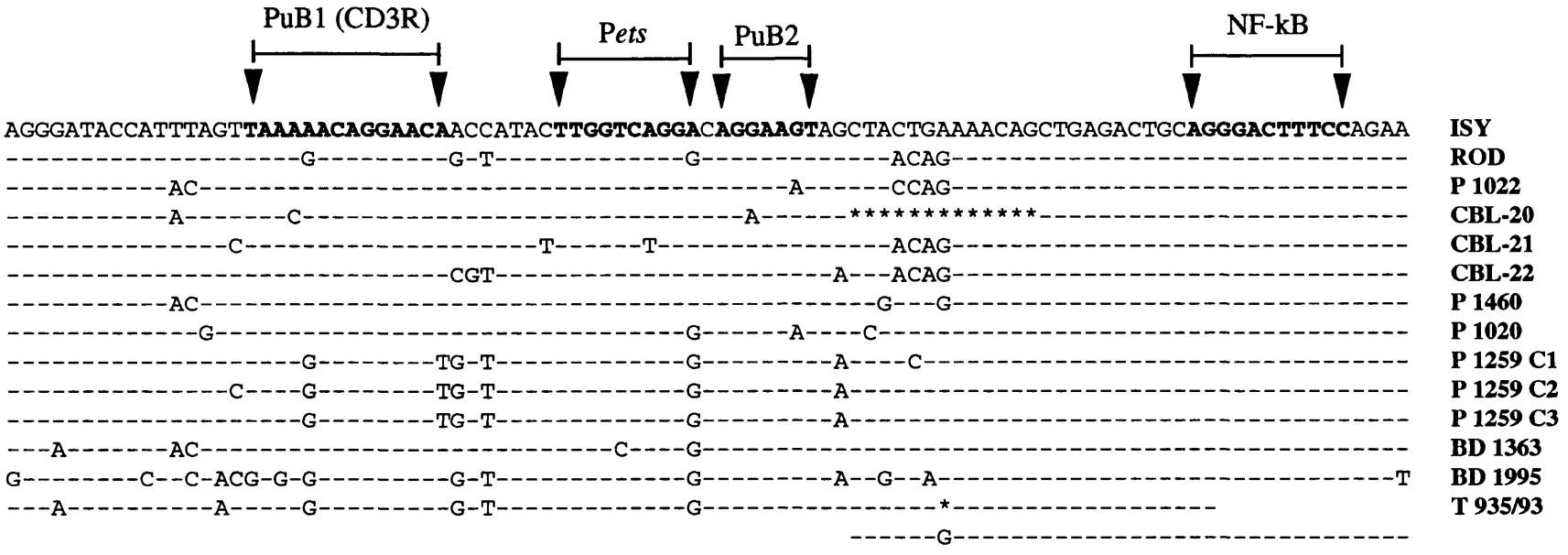


Figure 6.4. Sequence alignment of *in vivo* strain variation of part of the HIV-2 U3 LTR. The four *cis*-acting sequences defined for HIV-2 subtype A strains are indicated in bold. Nucleotide sequences of PCR-amplified DNA from laboratory isolates (HIV-2 ISY, ROD and CBL.20-22), patient-derived DNA (P1022, P1460, P1020 and P1239) and RNA derived from cDNA (BD1363, BD1995 and T935/93). The co-ordinates for this region numbered from the start of transcription in HIV-2 ROD are -189 to -94.

chapter. Very few changes in proviral sequences were identified with there being no significant differences between laboratory strains or patient-derived PBMC samples (P1022, P1460, P1020, P1239). The *cis*-acting elements appeared to be conserved where the proviral DNA levels were either high or low. Sequence changes found in these strains are most frequently G for A substitutions which appear to be consistently repeated but do not greatly affect the three defined sites upstream of the NF-κB site which remain largely unchanged. The exception to this is the PuB-1 (CD3) site of sample BD 1995 which exhibits sequence differences from the consensus. However, the largest changes occur between the PuB-2 and NF-κB sites in CBL-20 as a 13bp deletion and in T935/93 as a 25bp insertion. The function of this intervening region remains to be ascertained although it is possible that such changes in an otherwise highly conserved region may have an effect on the replication properties of the virus.

With the exception of CBL-20 and the PCR clones generated from patient sample P1259, all other sequences from laboratory strains (CBL.21/22/23), proviral DNA and viral RNA were derived from sequencing of bulk PCR product and therefore represent a consensus sequence for each analysed. In the case of the three samples from blood donors where plasma RNA provided the starting material, the sequences were particularly clean with few ambiguities. The autoradiograph from which these sequences were read is shown in Figure 6.5. Cell-free plasma RNA derived from plasma samples, reverse transcribed, PCR amplified and directly sequenced would therefore be the approach favoured in further studies.

6.b.4. Genotype analysis of HIV-2 strains.

Analysis of the U3 sequences obtained also indicated that there was a degree of conservation within these viruses. Furthermore, the *cis*-acting elements with the exception of the PuB-1 site in BD1995 showed little variation *in vivo*. Sequences were therefore aligned with HIV-2_{ISY} and compared with HIV-$2_{CBL20\text{-}22}$ and HIV-2_{ROD} (subtype A), and in a separate alignment with HIV-2_{D205}, HIV-2_{UC1}, HIV-2_{7312A} and HIV-2_{EHO} (subtype B) and finally with SIV_{mac251} and HIV-2_{F0784} (SIV/-like) as shown in Figure 6.6.

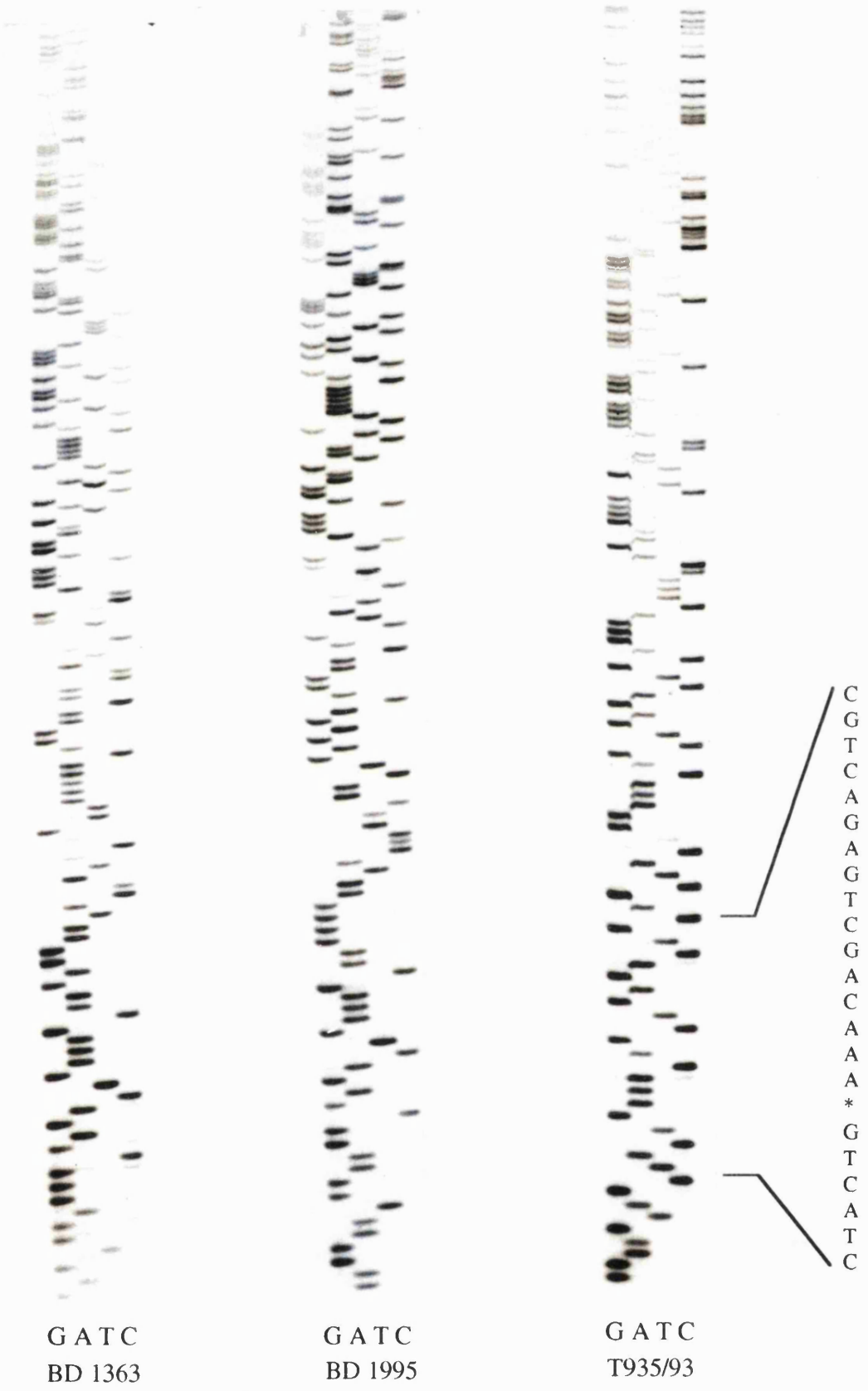


Figure 6.5. Autoradiograph of direct solid-phase sequencing of PCR products. Concensus sequences were obtained from a cDNA template from three patients. The duplicated sequence in patient T935/93 is indicated, which is located between the PUB-2 and NF-*k*B sites.

HIV-2 subtype A

```
CTGAAAGCAAGAGGGATACCATTTAGTTAAAA**ACAGGAACAACCATACTTGG**TCAGGACAGGAAGTAGCTACTGAAAACAGCTGAGACTGCAGGGACTTTCCAGAA ISY
----------A--------------------G---------G-T------------G------------ACAG------------------------------ ROD
----------------------AC-----------------------------------------A------CCAG------------------------------ P1022
....------------------A-------C-----------------------------A------************------------------------ CBL-20
--------T-----------------C--------------------T------T----------------ACAG------------------------------ CBL-21
-----------------------------------------CGT-----------------------A---ACAG------------------------------ CBL-22
.........------------AC-------------------------------------------------G---G------------------------------ P1460
............-----------G---------------------------------G------A----C---------------------------------- P1020
..........A--------------------G--------TG-T------------G---------A----C---------------------------------- P1259 C1
..........----------------C----G--------TG-T------------G---------A------------------------------------- P1259 C2
..........--------------------G--------TG-T------------G---------A------------------------------------- P1259 C3
--------------A-------AC-----------------------------C----G------------------------------------------- BD1363
----------G---A----C--C-ACG-G-G---------G-T------------G---------A--G--A-----------------------------T BD1995
--------------A----------A-----G---------G-T------------G----------------*------------------ T935/93
                                                                    ------G------------------------------
```

HIV-2 subtype B

```
CTAAAAGCAAGAGGGATACCTACAGATTAGGCAAGAGAC**AGCAGCATAAACAGGAACTAGCT**GACACTGCACA.AGAAGGAAACTAGCAGACACTGCAGGGACTTTCCAAAA D205
-------------------------C-----------------------------------------.---G----------T-----C----------------- UC1
--------------C-----------G---A--G--A-.-----------G-------------G-----T.----.------G-C--------------------G-- 7312A
--G-G---------A-----------G---A--G--A-T-A-----C---G----------A--------T-G-------------T--T--------.--------G-- EHO
```

SIV/SIV-like

```
CTAACCGCAAGAGGCCTT**********************************************TTAACATGGCTGACAAGAGGGAAACTCGCTGAGATAGCAGGGACTTTCCACAA SIV/251
------------------**********************************************-C----CA----------------AG------G------------------ FO784
```

Figure 6.6. Comparison of U3 sequences of HIV-2 subtype A, subtype B and SIV strains. The 40-44bp deletion in SIV and SIV-like viruses is shown. The subtype-specific primers used for selective amplification are shown in bold. Location of subtype A specific primer is in a relatively high region of conservation spanning the PUB-1 and pets sites. Dots indicate where either no sequence exists or was obtained.

From an analysis of the sequence data both from the published sequences and those generated in these studies, it became evident that putative groups of strains could be sub-divided according to their U3 sequence. The functional properties of the HIV-2 subtype A U3 LTR are discussed at the end of this chapter. However, for application to selective PCR analysis of HIV-2 and SIV strains these sequences were used to design a discriminatory nested PCR. Two additional primers were therefore synthesised, one corresponding to a concensus for subtype A strains and the other to subtype B strains as far as the degree of sequence homology within HIV-2 subtype A and HIV-2 subtype B strains would allow. The sequence information for HIV-2 subtype B viruses remains limited. Subtype-specific sequences identified were:

Subtype A: 5'-**ACAGGAACAGCCATACTTGG**-3' (9330-9349 HIV-2_{ROD})

Subtype B: 5'-**AGCAGCATAAACAGGAACTAGCT**-3' (9816-9838 HIV-2_{D205})

These sequences also broadly align with the deleted sequence in the SIV strains as shown in Figure 6.6. The following strategy was therefore adopted: following a preliminary screen for prototypic or SIV-(like) viruses where significant differences in size would allow their differentiation, further sub-division into subtype A and subtype B stains would be mediated using each specific primer in conjunction with the type-common biotinylated 3' primer. The specificity of these primer combinations was tested using the laboratory isolates for subtype A viruses as previously described (LAV-2_{ROD} and the CBL series) and also with HIV-2_{D205} which has a subtype B genome overall and HIV-2_{7312A} which has a subtype B LTR sequence. The results of this are shown in Figure 6.7. where selective secondary amplification was achieved with the respective primer combinations and HIV-2 subtype A and HIV-2 subtype B viruses discriminated according to their U3 LTR sequence.These primers were subsequently used to assess the relative proportions of subtype A and subtype B viruses in the sub-set of 40 PBMC DNA samples on which proviral load measurements had been made (Chapter 5).

All 40 proviral samples were subjected to type-specific amplification, and all yielded high intensity bands with the subtype A-specific primers but were negative with the subtype B

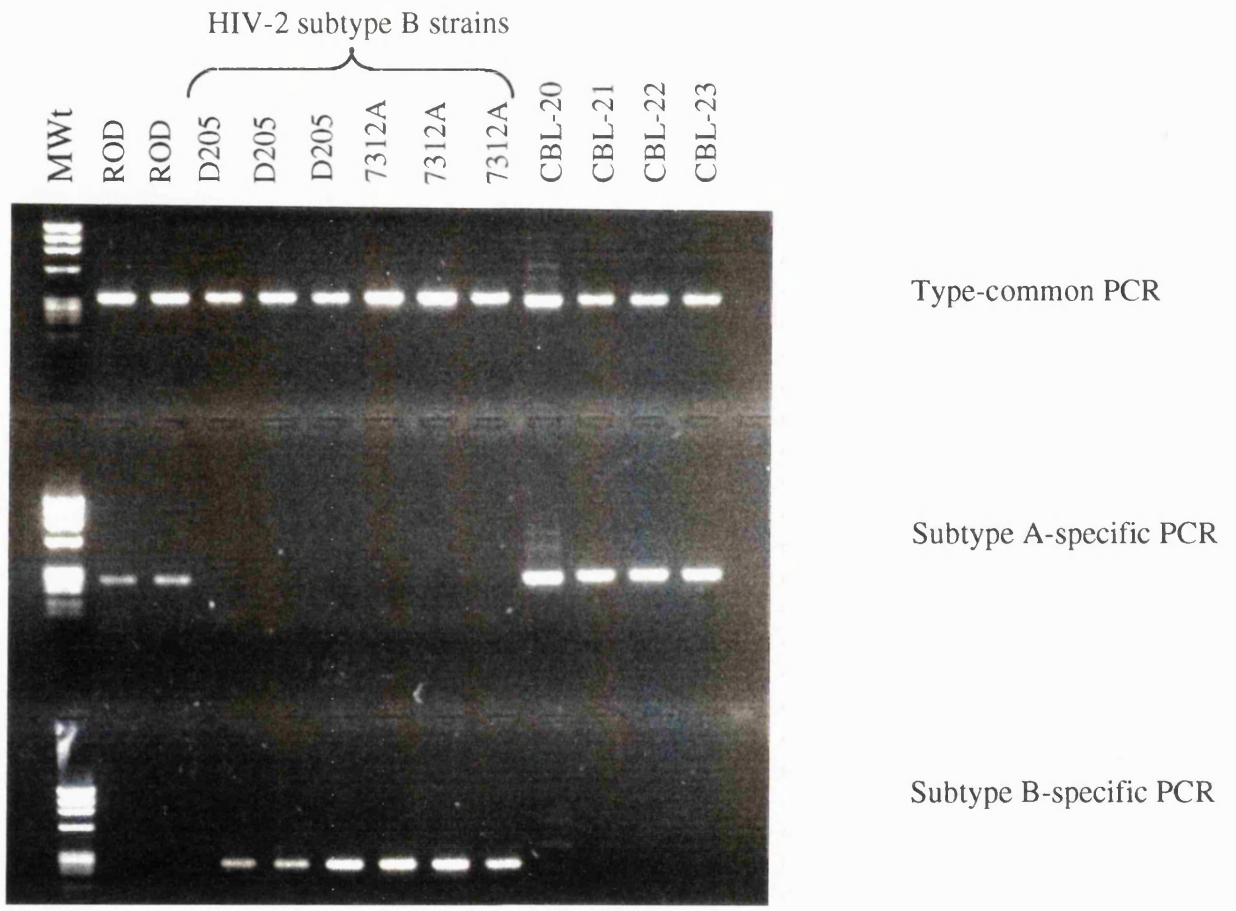


Figure 6.7. Genotype PCR analysis of HIV-2 subtype A and subtype B-specific strains. HIV-2 D205 and HIV-2 7312A have a subtype B LTR sequence and 10-fold dilutions were prepared from 10^3 to 10^1 copies. LAV-2 ROD (10^2 and 10^1 copies) and the CBL-series (10^2 copies) are all subtype A strains.

specific primers (Figure 6.8.). Application of these PCR tests to other clinical material including the RT-PCR-derived amplicons confirmed the presence of HIV-2 subtype A strains in these patients, according to their LTR sequence (Figure 6.8.). These studies demonstrate application of subtype-specific amplification of the HIV-2 LTR using clinical samples and as such represents a rapid means of identifying HIV-2 subtype A viruses, and differentiation from either subtype B or SIV-related viruses.

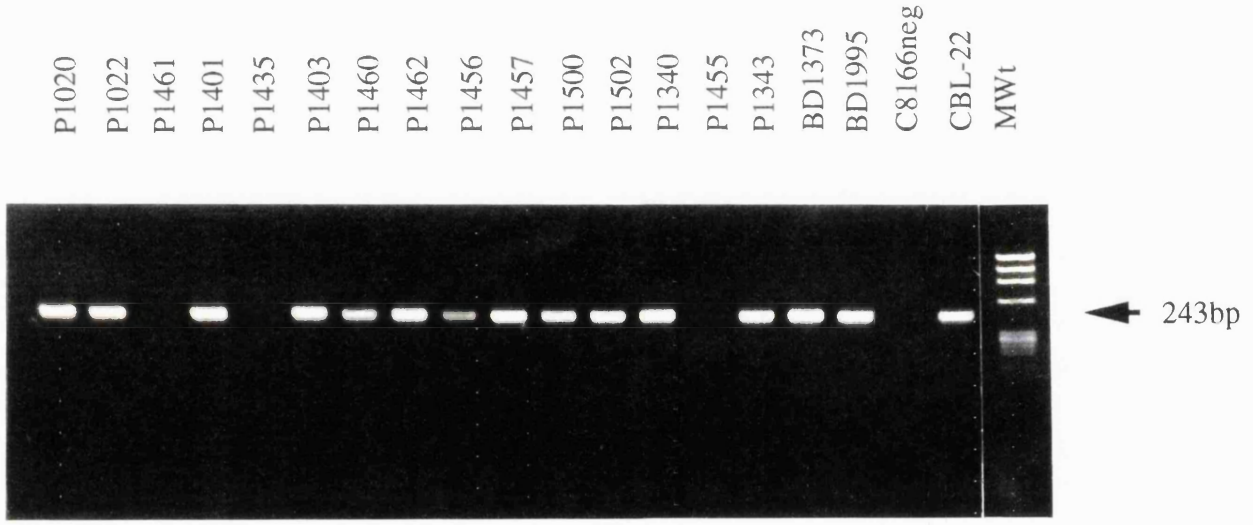


Figure 6.8. HIV-2 subtype A-specific amplification of PBMC DNA from Gambian HIV-2-infected individuals. Negative lanes (failure to amplify) were from HIV-2-uninfected individuals. Subtype B specific primers were negative with all samples tested. Amplicons for BD1363 and BD1995 were derived from cDNA.

6.c. Discussion.

The HIV LTR is responsible for controlling regulation of gene expression and in many respects functions as a T-cell activation gene, responsive to a wide range of cellular transcription factors (Gaynor,1992). The characterisation of these proteins and the specific sequences with which they interact have been studied in considerable detail for both HIV-1 and HIV-2. Much work has focussed on the U3 portion of the LTR, which as well as containing the classical enhancer and promoter sequences involved in transactivation, also contain sequence-specific binding regions for cellular factors. Differences in these sequences between HIV-1 and HIV-2 have been suggested to account, at least in part, for the different periods of asymptomatic infection observed for HIV-1 and HIV-2 (Markovitz *et al*, 1992) although analysis of these sequences *in vivo* has not been previously described.

Interactions between virus-encoded sequences and host cellular factors may therefore contribute to differences in pathology and natural history. The studies described in this section relate, in particular, to the U3 sequence in HIV-2 strains and compare the sequences from several patients at different stages of HIV-2-related disease (reflected by their proviral DNA and viral RNA levels) with the U3 sequences of the established Gambian strains CBL.20-23. The U3 region of the HIV-2 LTR was interesting to study from a number of perspectives. Firstly, this region differs between prototypic HIV-2 strains and many of the SIV strains since it is in this region where the 40-44bp deletion in SIV strains resides. A strategy of differentiating HIV-2 from putative SIV strains was therefore employed such that a difference in the electrophoretic mobility of these viruses could be used as an initial means of screening for SIV-related variants and for determining their relative prevalence in human populations.

The HIV-1 LTR can be distinguished from the HIV-2 LTR and has been extensively characterised (Gaynor, 1992). The stimulation of HIV-1 transcription has been shown to be largely, although not entirely, dependent on two κB regulatory elements which bind specific nuclear factors (NF-κB) important for the regulation of HIV gene expression (Nabel and Baltimore, 1987). These 10bp elements (GGGACTTTCC) are repeated twice in the HIV-1 enhancer component and are located immediately upstream of three Sp1

sites and the TATA box and have been implicated in T-cell activation, cytokine regulation and as intracellular messengers (Lenardo and Baltimore, 1989). For HIV-2, only one of these κB elements is present with the first immediately upstream from the Sp1 sites functionally inactive as a κB response element. It is possible that this second site in HIV-2 may be responsive to other cellular factors such as AP-3 (Tong-Starksen *et al*, 1990). At least one NF-κB site, however, appears to be functionally conserved in both HIV-1 and HIV-2. The NF-κB site is also important in HIV-2 gene regulation although other sequence-specific elements have been identified.

Activation of the HIV-2 enhancer is mediated through stimulation of the T-cell receptor complex and at least four *cis*-acting elements within U3 are involved in activation of the HIV-2 enhancer, which have been identified and mapped upstream of the TATA box and Sp1 sites. Only one of these elements is found in HIV-1 (the κB sequence) and all four are required for efficient induction acting synergistically with each other. The composition of the enhancer region of HIV-2 with all the relevant sites outlined shown in Figure 6.4. At least two of these are purine-rich sequences, containing a purine box (PuB) which are characterised by the motif 5'-AGGAA-3', termed PuB-1 and PuB-2. The PuB-1 sequence, originally termed CD3R, is purine-rich (TAAAGACAGGAACA) acting as a sequence-specific region corresponding to the CD3 part of the T-cell receptor complex (Markovitz *et al,* 1990 and 1992). PuB-1 has been shown to interact with the DNA binding protein *Elf*-1 which is a transcription factor belonging to the *ets* proto-oncogene family of transcription factors, related to the *Drosophila* E74 transcription factor (Leiden *et al,* 1992). A protein, designated PU-1, which recognises the purine-rich sequence 5'-GAGGAA-3' has been demonstrated to be a transcriptional activator that is expressed in both macrophages and B cells and which also has homology with the *ets* oncogene (Klemsz *et al,* 1990). The other purine-rich site in this region, termed PuB-2 which also contains the purine-rich pentanucleotide sequence (AGGAAGT) is also responsive to *Elf*-1. A further sequence has been identified which is proximal to the PuB-2 or *ets* sequence and has been named p-*ets* (TTGGTCAGGG). In conjunction with the single κB site, these represent the four elements within the enhancer of the HIV-2 LTR all of which are required for efficient activation of the HIV-2 genome and act together in a synergystic manner (Markovitz *et al,* 1992).

Arya and Mohr, (1994) present data in agreement with most of these findings and have emphasized the role of cell-specific transcription factors in HIV-2 gene expression. Deletion analysis of different sections of the U3 part of the LTR in both HIV-2_{ROD} and HIV-2_{ST} were compared in CEM, lymphocytic Jurkat, monocytic U937 and epithelioid HeLa cells. Constructs of HIV-2_{ROD} and HIV-2_{ST} were used because of their differing phenotypes, which was partly reflected in their LTR responsiveness. The HIV-2_{ROD} LTR was approximately twice as active as that belonging to HIV-2_{ST}. The TCAGG motif in the p*ets* sequence was identified as a positive regulatory element and a negative regulatory element containing an AGGAA motif was identified upstream of the PuB-1 site. This report suggests that one of the functions of *tat* is to abolish the effect of this negative regulatory element. Clearly, a complex set of interactions come into play in HIV-2 transcriptional regulation including *tat* activity but the essential role of the four *cis*-acting sequences has been corroborated by such studies. Hilfinger *et al*, (1993) have also studied the effects of these four essential elements in monocytes and found a similar role for all four elements in both mature and immature monocytes upon stimulation with phorbol esters. Recently, a fifth element has been identified between the PuB-2 site and the NF-κB sequence defined as a peri-κB site (GAAACAGCTGAGACTGC) which interacts with a monocyte-specific cellular transcription factor (Clarke *et al*, 1995). This report is the first to describe a HIV-2 enhancer sequence with monocyte specificity with no similar monocyte-specific element having been identified in HIV-1. The peri-κB site is also conserved in related simian lentiviruses.

The sequence for the peri-κB site corresponds, in part, to the deletion in CBL-20 and the duplication in T935/93. It seems possible, though uproven, that the absence of an intact peri-κB site in CBL-20 may have been the result of establishment and propagation of this virus in lymphocytic cell lines where the role of a monocyte-specific sequence would be greatly diminished. It is also possible that the reduced ability of CBL-20 to produce high titres of virus for antigen preparation in the early EIA studies (Chapter 3) may have been related to the deletion in the LTR. This remains speculative, however, since these changes did not occur in the other Gambian CBL isolates studied. It is also perhaps worthy to note that the CBL-20 isolate was derived from BM, a 20 year old male patient with clinically diagnosed 'slim disease' at the MRC clinic in The Gambia. CBL-20 clearly

caused cytopathic effects in virus culture and was, after all, the first HIV-2 isolate to be obtained from a Gambian individual by colleagues at the Chester Beatty laboratories. Over a period of time in our laboratory, this isolate became difficult to express at high levels in culture for antigen preparation and it is this feature which may be related to the possible introduction of a deletion within the LTR and not necessarily related to the *in vivo* properties of the strain originally infecting patient BM. Interestingly, where the level of circulating virus was measured by PCR amplification and quantification of cDNA, this was found to be at the lowest level of detectability in the serum of BM (1-5 molecules of cDNA) as determined by end-point dilution.

Similarly, the duplication of a second peri-κB sequence in T935/93 would indicate increased responsiveness of this virus in monocytes. However, the levels of cell-free virus in this individual were also low (equivalent to less than 1000 copies per ml of plasma) although this individual may have been recently infected. The fact that changes in this region have been demonstrated to occur *in vivo*, as cell-free replication-competent virus in plasma, would imply that changes in this region may be important in natural infection or related to attenuated virulence. The introduction of these changes into plasmid constructs and different cell-types would be needed to address and characterise their significance. However, the fact remains that these changes have occured not in the previously described *cis*-elements, but between three closely adjacent sequences and the fourth, the NF-κB site. Indeed, the elements upstream of the NF-κB site (the PuB-1, p-*ets* and PuB-2 sites) seem to be relatively highly conserved with most of the changes occuring between these regions, being single base-pair G for A mutations. The region with the highest level of polymorphism also occurs between these three elements and the NF-κB site, the sequence immediately upstream of it is also absolutely conserved in all isolates examined, suggesting some functional constraint. This region has now been identified as a peri-κB site with functional significance.

The parental strain and molecular clone of HIV-2_{NIHZ} has also been described as having a deletion within U3 which corresponds to part of the *nef* gene in the overlapping region. Although this represents a loss of some 227bp, this is upstream of the sequences required for genome activation and would appear to have no effect on the biological properties of

this isolate which is fully cytopathic and replicates with good efficiency (Zagury *et al*, 1990). No such dramatic changes in the *nef*/LTR region were observed with any of the isolates or clinical samples used in these studies.

In most SIV strains, with the exception of SIVagm, the 44bp deleted sequence broadly aligns with the three additional *cis*-acting elements in HIV-2, namely PuB-1, PuB-2 and the p*ets* sequence. These three elements appear to be relatively conserved amongst most HIV-2 strains, at least those with a subtype A LTR. Exceptions to this include the SIV-like viruses and HIV-2_{D205}, HIV-2_{UC1} and HIV-2_{EHO} which are also distinct from the bulk of HIV-2 strains having a subtype B LTR. The fact that these sequences are not present in SIV but play an essential role in HIV-2 activation and replication would imply that there are central differences between HIV-2 and SIV activation mechanisms and possibly subtype B viruses. Transcriptional activity of different strains of SIVmac (SIVmac239 and SIVmac251) have been compared which replicate in both lymphocytic and macrophage cell lines (Anderson and Clements, 1991). Differences in sequences and possible functions of these two strains within the U3 region were observed although no obvious differences in biology were attributable to these. Biological studies of subtype B viruses remain to be performed to elucidate possible different mechanisms of activation. Interestingly both HIV-2_{D205} and HIV-2_{UC-1} are characterised by their lack of cytopathic effects on T-lymphocytes, although the CD4 antigen is implicated in infection and both isolates are biologically similar in their ability to establish good growth in macrophages.

Differences in the biological phenotypes of HIV-2 strains, however, as described in section 1.b.2.ii. would suggest that no single factor or property is responsible for the biological variation observed but that an interplay between differing elements is involved in a dynamic process. However, by amplification of a region where there are sequence-specific differences involved in cellular/viral control pathways and using a combination of sizing by agarose gel electrophoresis and differential PCR, it was possible to differentiate HIV-2 subtype A, subtype B and SIV viruses from each other on the basis of their LTR sequence.

The samples analysed from The Gambia were all identified as being subtype A strains, even those with extremely low virus loads. This would suggest that those patients with low virus loads and high CD4 counts may have been recently infected with subtype A strains which would with time lead to severe immunodeficiency and AIDS. Amplification of all HIV-2 DNA samples with the subtype A-specific primer indicates not only that subtype A strains predominate in the samples analysed collected in The Gambia, but also that as a result of the high efficiency of amplification with this primer combination, the PuB-1 and p*ets* sequences which were used for subtype A-specific priming are also highly conserved *in vivo*.

The principal application of this approach to genotyping HIV-2 strains would be to apply subtype-specific differential nested PCR on a population basis and to assess the relative proportions of these subtypes circulating in West Africa. This may be viewed as an alternative and supplemental approach which would complement more detailed phylogenetic studies. These would be required to determine whether the LTR sequence was a reflection of the overall subtype classification of the genome based upon conventional sequence analysis of structural genes and for further refinement of genotype. Comparisons where differences in the natural history of HIV-2 infections have been described could be more easily performed and provide a means of identifying viruses which do not have the classical regulatory sequences typical of the majority of HIV-2 subtype A strains identified to date.

Chapter 7.

General Discussion.

Infection with either HIV-1, HIV-2 or in some cases both viruses is now widely considered to be causally linked to AIDS in humans (Schecheter *et al*, 1993; Romieu *et al*, 1990). These related yet distinct lentiviruses have represented two foci of the epidemic, particularly when viewed from an African perspective. HIV-2 has always been strongly linked with West Africa and until relatively recently has been largely confined to countries of the region. The impact of HIV-2 in global terms has therefore been limited and HIV-2 has assumed a less important role than HIV-1. However, the existence of a second, related lentivirus in human populations merits attention and scrutiny and it is perhaps because the effects of HIV-2 appear to have been less than those observed for HIV-1 that the relative pathogenicity of HIV-2 in humans deserves special attention.

At the onset of these studies, very little firm evidence had been accumulated relating to the natural history, epidemiology and evolution of disease in HIV-2-infected individuals. Indeed most of the reports of HIV-2 infection were in the form of case histories of individual patients in whom the virus had been detected, some of whom were identified as having an AIDS-like illness. It was also suggested, however, that HIV-2 was associated with a much longer period of asymptomatic infection than was being described for HIV-1. This led to two differing viewpoints relating to the relative pathogenicity of HIV-2. Clinical investigations at MRC Fajara led to some of the first reports showing that HIV-2 could be fully pathogenic in humans resulting in AIDS. Clinical and epidemiological studies in other parts of Africa, in particular Senegal, suggested that HIV-2 was not pathogenic for humans, and not primarily associated with HIV-related disease (Kanki, 1987). However, even as more HIV-2-related AIDS cases became identified across West Africa, it was becoming increasingly apparent that although HIV-2 was capable of acting as a pathogenic retrovirus, the effects of infection with HIV-2 were less compared with HIV-1. The points raised in this discussion attempt to address some of these apparent differences in the light of studies performed since this time both in terms of the development of the HIV-2 epidemic in West Africa and an

increased understanding of the biological and genetic differences between HIV-1 and HIV-2 and their evolutionary relationship with simian lentiviruses.

If nothing else, HIV-2 provides a different perspective from which to view the global AIDS pandemic and comparative studies of the two infections, particularly in view of changes in the prevalence of HIV-1 in West Africa, are important. One of the first goals in such studies is a secure means of identification and differentiation of the two viruses. This has been an important objective in collaborative studies in Fajara and has provided a basis for subsequent virological and clinical investigations. The studies described although performed only on samples obtained from The Gambia have been related to the wider scenario of the HIV-2 epidemic.

7.1. Diagnosis of HIV-2 infection.

Serological studies covered in Chapter 3 indicate that competitive assays for anti-HIV-2, using either culture-derived native viral antigens or recombinant proteins, serve as a reliable and sensitive means for detecting HIV-2-specific antibody. Furthermore, these assays were demonstrated to be equally efficient at anti-SIV detection showing comparable sensitivity on sera obtained from different monkey species. This may be significant when identifying SIV-related strains in humans since these viruses have been demonstrated to exist in humans representing part of the diversity of HIV-2-related strains. Application of competitive EIAs would therefore not preclude the detection of SIV-like variants in humans.

This may be viewed in context of the identification of the widely variant HIV-1 subtypes, in particular variants detected in the Cameroon (Gurtler *et al,* 1994; Nkengasong *et al,* 1994). DNA sequencing and phylogenetic analysis of isolates have shown them to be related to HIV-1 although they have been designated HIV-1 subtype O indicating their exclusion from eight other groups of HIV-1 (subgroups A-H) which encompasses the known genetic variability of HIV-1 isolates worldwide. Patients infected with subtype O variants react only weakly, if at all, in most serological assays and therefore would be unlikely to be diagnosed as being HIV-infected (Loussert-Ajaka *et al,* 1994). This has caused considerable concern in the field of HIV diagnostics and attempts have been

made to include representative antigens from subtype O variants in serological assays in order to ensure their detection. A similar problem exists for HIV-1 genome detection, where PCR primer sets are known not to react with equal proficiency with genetically diverse strains. This has highlighted the problems of diagnosing viral infections where the spectrum of variants, or potential for them, is large and places constraints on diagnostic tests in only being able to detect reliably the range of viruses already known. Weakly reactive samples, in either serological or genome amplification assays, may therefore be as a result of low-level non-specific reactivity or a divergent virus exhibiting weak but specific cross-reactivity with a distant relative.

In the case of HIV-2, a similarly broad range of genotypes may exist, particularly if this includes the SIV-like HIV-2 viruses. The approach adopted in these studies has been to use a competitive EIA for antibody detection as described in Chapter 3 and nested PCR using conserved regions of the genome for HIV-2 DNA detection as described in Chapter 5. In both cases, anti-SIV (SIV_{mac}, SIV_{sm} and SIV_{agm}) and nucleic acid sequences (SIV_{mac} only) were used to investigate the reactivity with both types of assay. The competitive EIA responded well in terms of sensitivity to antibody raised to each virus-type and can be taken as an indication of the broad detection range of the HIV-2 assay, including SIV strains. Therefore, if any related viruses were to appear in the human populations studied their detection is likely to be ensured. Similarly, the LTR PCR primer set was also tested on SIV DNA, and strong reactivity indicated that SIV-related variants of HIV-2 would also be detected. The comparable sensitivity of the diagnostic LTR primer set with the HIV-2 and SIV strains tested and the strong concordance of PCR-amplifiable DNA from seropositive individuals, would indicate that these primers serve as reliable targets for PCR amplification. While it is virtually impossible to predict the frequency of occurence of widely divergent viruses, if the SIV strains are considered to be at one end of the spectrum of HIV-2 viruses, a reasonably high level of security may be placed on the detection systems established.

Concerted attempts to measure p26 antigen levels in the serum or plasma of HIV-2-infected individuals were not attempted although the recombinant p26 antigen and anti-p26 serum developed (Chapter 4) could be applied in such studies. It is perhaps worth

bearing in mind, however, that where the prevalence of HIV-1 p24 antigen has been compared in African and European HIV-1-infected individuals, differences in prevalences of 4% and 64% for detectable p24 antigen between the two groups was observed which correlated with low levels of anti-p24 in the African individuals (Kaleebu *et al*, 1991). The prevalence of HIV-2 p26 antigen in patients at different stages of disease, however, remains unknown. This may be the focus of future studies and may be a useful predictor for progression and transmission when combined with HIV-2 RNA detection assays. Where HIV-2 RNA was detectable in blood donors (Chapter 6), the levels were low and an analysis of cell-free HIV-2 levels in cross-sectional studies remains to be performed. The use of empirically derived HIV-1 and HIV-2-specific assays have been used and continue to be used in studies in Fajara. The increase in the prevalence of HIV-1 in The Gambia and in other parts of West Africa in recent years gives cause for concern and the differentiation and identification of the infecting virus-type assumes a heightened significance.

7.2. Relationship between virus load and virus subtype.

Virus load data, generated by PCR amplification and quantification of proviral DNA, showed a strong inverse relationship between virus load and immunological status (Chapter 5). Aspects of this work complement the findings of Simon *et al*, (1993) where the use of culture-based sytems showed the levels of virus in HIV-2-infected patients to be similar to those in HIV-1-infected patients where the CD4 counts were below 200. Several studies have shown that it may be difficult, if not impossible, to derive a viral isolate from asymptomatic patients with high CD4 counts. We found, however, that it was possible to obtain a PCR signal in such circumstances and therefore to obtain virological measurements. In other studies of West African HIV-2-infected patients it was also found that healthy individuals who were clearly infected, as determined by serological investigations, would not yield virus by culture but harboured HIV-2 viral sequences (Gao *et al*, 1992). Broad genetic diversity of HIV-2 in humans has been described and at least five different subtypes (A-E) of HIV-2-like viruses have now been proposed (Gao *et al.* 1994). Several of these strains have been identified as being more closely related to strains isolated from the sooty mangabey monkey which is indigenous

to West Africa providing the strongest evidence yet that HIV-2 infection in humans is the result of a zoonotic transmission. The HIV-2_{F0784} virus has been classified as subtype D.

To address further the question of differing subtypes of HIV-2 strains on a population basis, PCR assays were developed using the extended *nef*/LTR nested primers and applied to the samples tested for proviral load. The results of the PCR and direct sequencing analyses of the Gambian HIV-2 samples, (Chapter 6) indicated that no SIV/HIV-2_{F0784}-like strains were present. Viral sequences identified from patients with few or no clinical symptoms with low proviral loads and those with severe immunodeficiency and high proviral loads were all of subtype A. This suggests that the patients on whom proviral load measurements were made were a cross-section of individuals infected exclusively, as far as the genotyping studies allow, with subtype A viruses. The distinction can be made that given the restrictions placed on interpretation of U3 LTR selective PCR, no subtype B or SIV-like variants were present.

The value of subtyping HIV-2 strains on a population basis remains to be assessed, however, and may be an over-simplification of the situation. Gao *et al*, (1992 and 1994) have also demonstrated that the HIV-2 genome exists as a mosaic consisting of different subtype recombinants where divergent viral strains have spread in the same population. Sequence analysis of the different structural genes of *gag*, *pol* and *env* may result in the viruses clustering in a different position in phylogenetic trees. HIV-2_{7312A}, for example, is subtype B for most of the genome, including the distinctive LTR sequences but has a subtype A envelope. For the HIV-2_{CBL} series of isolates studied, however, the LTR sequence reflected that of one of the structural genes (*gag*), both identifying with subtype A (Chapters 4 and 6). The application of DNA-amplification techniques to the study of HIV-2 *in vivo*, however, is likely to remain an important area of study given the limitations of culture-based methodologies.

Where diagnostic PCR has been performed on patients with virus-specific antibody for HIV-2, amplification has been unsuccessful in some cases (Grankvist *et al*, 1992; Simon *et al*, 1993). It is possible that this could be due to HIV-2 variants although these studies both used nested PCR primers located in conserved regions of the LTR, and a low

proviral load remains the most likely explanation for failure to amplify. Since it was subsequently demonstrated that the Gambian strains tested for proviral DNA were all subtype A, this may account in part for the improved detection rate of nested PCR described here compared with other groups. There were no samples from the Ivory Coast, for example, in our studies which may have a higher proportion of subtype B viruses than other parts of West Africa. However, in the current Gambian study, although subtype B variants were not identified, at least on the basis of their U3 LTR sequence, the extended *nef*/LTR primer sets were demonstrated to detect the LTR of both HIV-2_{7312A} and HIV-2_{D205} with good efficiency. Therefore if such viruses were encountered in clinical samples it is also likely they would also have been detected.

Where subtype B viruses (or where a subtype B LTR has been identified as in the case of HIV-2_{7312A}) have been identified, they have been not obtained from individuals from the western part of West Africa but from more central and eastern West Africa such as Nigeria, Ghana and the Ivory Coast. HIV-2_{D205}, HIV-2_{UC-1}, HIV-2_{GH-2}, HIV-2_{EHO} (section 1.b.2.ii) were isolated from individuals from the Ivory Coast or Ghana and HIV-2_{JA} HIV-2_{ON} and HIV-2_{FT} have also been genetically characterised as subtype B viruses obtained from individuals from this region (Gao *et al*, 1994). Recently, SIVsm isolates from sooty mangabeys have been made in the Ivory Coast (Peeters *et al,* 1994b) which further indicates the diverse nature of animal lentivirus infection and may be linked with the apparent higher proportion of subtype B viruses in this geographical region. The analysis of samples from different areas of West Africa would be interesting to pursue, although the detection of divergent genotypes compounded by low infectivity of such strains may place extreme limitations on detection systems.

Gao *et al*, (1994) also propose that subtype A viruses are most likely to produce a positive culture, irrespective of the time-period or stage of disease of the patient from which they were derived. Patient 6041K, for example, in that study had high CD4 counts in excess of 500 cells/mm^3, and yet an isolate was obtained. The cross-sectional study of proviral DNA load in The Gambia (Chapter 5) indicated that HIV-2 is capable of replicating to very high levels *in vivo* and that, based upon LTR sequences, these were all subtype A viruses. The concept of subtype-specific virulence, attenuation and reduced

pathology of certain HIV-2 subtypes (C-E) proposed by Gao and colleagues is attractive although further studies on asymptomatic individuals are required to substantiate this. Subtype A viruses may be more transmissible since subtype A, and subtype B viruses to a lesser extent, still represent the majority of HIV-2 strains identified to date with members of other subtypes (C-E) few and possibly restricted to particular geographical areas within West Africa. In a study of HIV-2 *env* sequences comparing laboratory isolates from The Gambia and Guinea Bissau, preliminary data has hinted at the possiblity of separate genotypes existing within the HIV-2 subtype A group of viruses (Breuer *et al*, 1995). The significance of these data in terms of the clinical outcome of HIV-2-infected individuals, however, remains to be seen although subtype A viruses may be genetically more diverse than other HIV-2 subtypes.

The application of the PCR-based genotype or subtype method described in this thesis may provide a means of ascertaining which types of HIV-2 strains are present *in vivo*, particularly since culture-based studies for asymptomatic HIV-2 infection are hampered by the inability to culture virus. The relative proportion of non-prototypic strains in populations of HIV-2-infected individuals remains to be determined and therefore a rapid means of identifying such strains would be useful. This may be viewed as an alternative to sequencing large sections of the HIV-2 genome and a rapid subtyping approach would also complement more detailed sequencing and phylogenetic studies. Clearly more detailed studies on diverse populations of HIV-2-infected patients need to be performed, preferably where epidemiological differences have been noted. A combined approach of subtype analysis and measurement of viral load may provide the most rapid representation of the *in vivo* situation for population-based analyses of HIV-2 infection.

7.3. Properties of Lentiviral LTR sequences.

The Long Terminal Repeats of animal retroviruses play an important role in the life cycle and virus biology, although there are often uniquely specific sequences which characterise the respective LTRs of different retroviruses and exert a profound effect on transcriptional control, interacting with the cellular environment. In some oncoviruses, enhancer sequences have been implicated in inducing cell proliferation in different types of leukaemia (Golemis *et al*, 1989). Regulatory motifs in the LTRs of HIV-1 (Chang *et*

al, 1993) and SIV (Renjifo *et al*, 1990; Anderson and Clements, 1991) have been studied relating to the role of *cis*-acting sequences within U3. Sequence differences within U3 for different viruses appear to alter basal trancriptional activity and be particularly relevant with respect to response with the cellular environment.

In HIV-1 infection, the NF-κB element plays an important role in stimulating transcription by binding the NF-κB moiety which exists when free, as uncomplexed p50-p65 heterodimers (Gaynor, 1992). Transcription of the HIV-1 LTR is boosted by NF-κB moieties acting together with *tat* to initiate transactivation of the genome in target cells, for example in Jurkat T-leukaemia cells where mutation of the NF-κB sites in HIV-1-infected T-cell lines has been shown to abolish induction of the LTR (Liu *et al*, 1992). Differences in the NF-κB family of proteins and their binding properties may also be related to the wide spectrum of HIV-1 gene expression in varying cell types (Oakes *et al*, 1994). Earlier studies indicated that reactivation of HIV-1 from a period of clinical latency may also be related to properties of NF-κB binding and subsequent cytokine activation (Lowenthal *et al*, 1989) which may also have an effect on a wide variety of cell types, including glial cells (Atwood *et al*, 1994). The NF-κB elements in HIV-1 can therefore act in an immunomodulatory capacity and are responsive to cytokines, in particular TNF-α (Osborn *et al*, 1989). The effect of TNF-α on the HIV-2 LTR, however, appears to be markedly reduced possibly due to the lack of a second NF-κB element (Arya, 1991). Responsiveness to TNF-α in HIV-2, however, does seem to be mediated by the κB element, and mutation of other *cis*-acting elements have no effect on the response to TNF-α (Hannibal *et al*, 1993).

Enhancer activity in HIV-1 infection also involves other *cis*-acting elements which also play a crucial role in HIV-1 genome activation (Gaynor, 1992). However, the principal difference between HIV-1 and HIV-2 in this respect is that HIV-1 transcription can be mediated more by NF-κB alone, whereas in HIV-2 additional factors appear to be essential for transactivation. This powerful enhancer element plays a different role in HIV-2 transactivation acting in concert with other cis-acting elements (Chapter 6). Interestingly, duplication of a second NF-κB sequence in SIV$_{smmpbj14}$ has been linked with the pathogenicity of this virus. The SIV$_{smmpbj14}$ isolate exhibits unusual biological

properties and was obtained following experimental transmission of a virus (SIVsmm9), originally isolated from an asymptomatic sooty mangabey monkey to a pig-tailed macaque (Fultz *et al*, 1989). When either pig-tailed macaques or sooty mangabeys are infected with this virus, a fatal disease is produced within eight days of inoculation (Dewhurst *et al,* 1990). One of the findings from DNA sequence analysis of this isolate was that it contained a 22-base pair duplication in the enhancer region (U3) of the LTR and a 15bp (5 amino acids) duplication of part of the envelope surface glycoprotein.

Subsequent studies attempting to link the additional NF-κB site with the cause of the acute pathogenicity of this virus were initially inconclusive (Dewhurst *et al*, 1992). Determinants within the *env* and *gag* genes have been shown to play an important role in the ability of this virus to induce acutely pathogenic effects within a short time period and the combined effect of the duplicated sequences in LTR and *env* may be essential to its enhanced pathogenicity (Novembre *et al*, 1993; Courgnaud *et al*, 1992). Further studies, however, have demonstrated a clear link between the additional NF-κB element and the high degree of pathogenicity which characterises certain clones of SIVsmmPBj14 (Dollard *et al*, 1994). Duplication of 22bp of LTR containing the enhancer sequence was related to a higher basal transcriptional activity when compared with prototypic SIVsmm LTRs and effects on cellular stimulation showed a two to three-fold increase in responsiveness.

The involvement of the NF-κB enhancer in HIV pathogenesis is therefore of interest since it not only acts as a powerful element in the regulation of gene expression mediated by the specific cellular factor NF-κB but has also been demonstrated to interact with specific cytokines such as tumour necrosis factor-α (TNF-α) and some interleukins (IL-1 β and IL-6). It has been proposed that TNF-α is involved in AIDS pathogenesis by modulating activation of the HIV-1 genome by acting on the NF-κB sites. Since HIV-2 has a different mode of activation with only one NF-κB element, the role of TNF-α and other cytokines may be different to that described for HIV-1. Comparative studies of HIV-1 and HIV-2 plasma cytokine levels in African patients, demonstrated a lower TNF-α response was associated with a lower viral load and higher CD4+ count than in HIV-1 infection (Chollet-Martin *et al*, 1994). The role of TNF-α remains controversial although

modified activation of cytokines on the HIV-2 LTR may contribute to the overall differences in HIV-1 and HIV-2 disease induction. If viral load does correlate with disease progression, it may be postulated that virus production is related to activation of HIV-2 proviruses the control of which may be under the influence of different factors than in HIV-1. The regulatory mechanisms controlling activation of HIV-2 in resting T cells may therefore have a bearing on disease outcome, not only in specifying tissue-tropism but also in determining the rate at which virus replication proceeds, modulated in turn by interactions with the immune system.

LTR U3 sequences have also been studied for other immunodeficiency viruses with respect to pathology (Clements and Payne, 1994). The LTR of BIV, has been studied in terms of regulation of replication and has been reviewed by Gonda *et al,* (1994). Initiation, termination and enhancement signals for transcription are contained in the U3 region of the LTR in common with other immunodeficiency viruses. The BIV LTR contains only one Sp1 site and one NF-κB which is relatively distal from the TATA box and may be related to the relative pathogenicity of BIV, influencing the rate of viral replication (Carpenter *et al,* 1993). The core enhancer region residing within the U3 LTR has a profound effect on promoter function and elements within this region play an essential role in upregulating viral expression as is the case for other immunodeficiency viruses (Pallanesch *et al,* 1992). In the BIV model, these sequences may also be involved in the varying expression in different cell types whereby relative expression is dependent on the species and the tissue or cell-line of origin, having major implications for temporal expression *in vivo*. Interestingly, BIV has modified sequences within the LTR and, although it has a wide range of *in vivo* target cells it is immune cells of the monocyte/macrophage lineage that appear to be most susceptible to infection (Carpenter *et al,* 1992). FIV also contains unique sequences in the LTR, described following characterisation of PCR clones, which have a major influence on the basal promoter activity in a variety of cell lines (Thompson *et al,* 1994). The FIV LTR shares a number of common features with the LTR elements of EIAV and MVV, neither of which cause immunodeficiency-related symptoms in their respective hosts.

The properties of the LTR have also been studied in these other animal lentiviruses. MVV and CAEV infect monocytes, which only produce productive virus when they mature into macrophages following activation of latent provirus. This process of maturation and activation is mediated in part by the transcription factors *fos* and *jun* which interact with the AP-1 site in both the promoters of cellular genes and the same sequence in the visna-virus LTR, leading to the production of progeny virus (Shih *et al*, 1992; Clements *et al*, 1994). In EIAV, different U3 sequences have similarly been implicated in transcriptional control and determination of cell specificity with a number of *cis*-acting elements, including an AP-1 and a PB-1 (*ets*) binding site, having been described (Carvalho and Derse, 1993; Carvalho *et al*, 1993, Payne *et al*, 1994). The expression of *tat* is also central to gene regulation in EIAV (Maury *et al*, 1994). These studies all suggest that sequence variation in the EIAV LTR may directly influence virus replication in different cell types. These viruses do not cause classical CD4+ depletion characteristic of immunodeficiency viruses but are classified within the *Lentivirinae*. Comparisons of transactivational control mechanisms of the various members of this group of related yet distinct retroviruses may help to understand more fully the pathogenic processes involved in lentiviral infection.

7.4. Retroviral origins: why is there an AIDS pandemic?

Retroviral-like elements have been described in both prokaryotic and eukaryotic cell types. The similarity of transposable elements with retroviral components (*gag* and *pol*-like sequences) has given rise to the notion that retroviruses may have evolved from cellular protoviruses, themselves derived from retroviral-like elements (Temin 1970 and 1980). Reverse transcriptase was probably one of the first enzymes in evolutionary history and retroviral-like elements may also have had an impact in the evolution of gene expression in eukaryotic cells, with specific sequences having arisen as a result of the insertion of retro-transposable elements (retrotransposons) into cellular genes (Robins and Samuels, 1992).

Retroviruses, hepadnaviruses and caulimoviruses represent three major groups of viruses identifiable today which exhibit reverse transcriptase activity at some point in their life cycle. Reverse transcriptase sequences have been used to determine the phylogenetic

relationship between retro-elements from animals, plants, protozoans and bacteria (Xiong and Eickbush, 1990). Cauliflower mosaic virus (CaMV) is a plant DNA virus which replicates via an RNA intermediate whereby synthesis of CaMV DNA can be partly inhibited by ribonucleases and actinomycin D (Pfieffer and Hohn, 1983). Members of the *Hepadnaviridae*, including hepatitis B virus, also replicate by reverse transcription (Summers and Mason, 1982). In yeasts, Ty elements exhibit transposition within the yeast genome. The mechanism through which this occurs is via an RNA intermediate such that Ty sequence information flows from DNA → RNA → DNA (Boeke *et al*, 1985).

Retrotransposons exist which both possess and lack LTR sequences. Those which lack LTRs encode a functional reverse transcriptase and replicate via an RNA intermediate. It has been proposed that the non-LTR retrotransposons are the likely progenitors of modern retroviruses and LTR retrotransposons (Eickbush, 1992). The *copia*-movable genetic elements in *Drosophila melanogaster* have been shown to have LTR sequences which exhibit a high degree of homology with the LTRs of avian leukosis virus (Kujimiya *et al*, 1983). Infection of *Drosophila* with a retrovirus from which the current avian leukosis virus was derived seems to be the most likely explanation. The evolutionary relationship between *copia*-like elements in *Drosophila* and vertebrate retroviruses has also been shown by identifying the presence of *gag, pol* and *env*-like open reading frames including a coding sequence for a reverse transcriptase-like enzyme (Saigo *et al*, 1984).

Reverse transcriptase sequences are therefore widely distributed in the animal and plant kingdoms. Modern, infectious vertebrate retroviruses, found mainly in birds and mammals, are thought to be a relatively recent occurence possibly within the last 100 million years which are related to ancient forms of retroviral-like sequences now exhibiting horizontal transmission (Doolittle *et al*, 1989; Doolittle and Feng, 1992). Integrated endogenous viral sequences may have given rise to infectious retroviruses identifiable today (Coffin, 1985). One endogenous retrovirus, human endogenous retrovirus (HERV-C), has copies integrated in the genome in the same places of both humans and chimpanzees indicating that it was introduced into the genome before the divergence of humans and chimpanzees, about eight million years ago (Steele *et al*,

1986). HERV-K contains open reading frames suggesting a functional provirus, but also possesses numerous translation stop codons such that proviruses are incapable of forming a functional viral particle (Doolittle *et al*, 1989). One feature which distinguishes an exogenous retrovirus from either a retrotransposon or an endogenous retrovirus, however, is the ability to derive a functional envelope protein thereby allowing escape from the cell. In higher vertebrates the evolution of modern retroviruses may be a reflection of a more sophisticated immune system, resulting from an interaction between components on the virus envelope and the host cells' receptor which plays a key role in retroviral biology (Weiss, 1993). Infectious retroviruses may have arisen and disappeared throughout evolutionary history, although the extent and severity in terms of human disease may have been limited to outbreaks in isolated populations. Infectious retroviruses in different species of non-human primates appear to be tolerated and horizontal transfer from one species to another may have led to pathogenic variants arising in the new host. This may account for both the HIV and HTLV group of viruses, which are members of different sub-families of the *Retroviridae*, where simian counterparts have been identified.

The evidence for a simian origin for HIV-2 in humans, however, is perhaps more convincing than for HIV-1 at the current time. The existence of SIV-like viruses present within the same phylogenetic group as HIV-2 has been interpreted as a reflection of past and possibly continuing cross-species transmission events between simian hosts and humans (Gao *et al*, 1992). Infection of humans with a virus tolerated by feral sooty mangabey monkeys, which are indigenous to West Africa and are hunted for food, may have resulted in transfer of infection at some point in the past. Humans are susceptible to infection from animal lentiviruses, demonstrated by transmission of SIV to a laboratory worker in the United States (Khabbaz *et al*, 1994) and SIV is capable of adaptation to human cells (Hirsch *et al*, 1989b).

An alternative candidate for a simian origin of HIV-2 is the African green monkey. SIV_{agm} viruses are also tolerated by their hosts, and phylogenetic analysis of the genomes of each of the sub-species of African green monkeys, the sabaeus, grivet, vervet and tantalus monkeys, indicate these viruses to be species-specific (Allan *et al*, 1991).

Co-evolution of each virus within its respective host where the virus has been tolerated by the immune system is thought to have occurred. Phylogenetic analyses of the SIVagm group of viruses has futher demonstrated the existence of mosaic genomes in lentiviruses and has confirmed the close correlation between the SIVagm isolates and the species of origin (Jin *et al*, 1994). Of the four African green monkey species, the sabaeus monkey is found in West Africa and SIVagmsab also exhibits a mosaic genome structure, parts of which can be linked with the HIV-2/SIVsm lineage (Jin *et al*, 1994). SIVagmsab cannot therefore be ruled out as a possible progenitor for HIV-2-like viruses or transmission between different primates in the wild. Certainly, the high degree of epitope recognition of the SIVagm sera in the HIV-2 competitive EIA (Chapter 3) was similar to that observed in natural HIV-2 infection. Any direct causal relationship between the SIVagm group of viruses and human lentiviral infection, however, remains to be established although ancestral origins of the primate lentiviruses may be linked to the *Cercopithicus* monkeys, where horizontal transmission between different monkey species may have occurred (Sharp *et al*, 1994).

While the appearance of HIV in human populations seems most likely the consequence of an introduction of a lentivirus, most likely from simian hosts, the course of the AIDS pandemic may be attributed to social and demographic factors as much as virological ones. The HIV-1 pandemic and the HIV-2 epidemic in West Africa have been viewed as relatively recent phenomena (Myers *et al*, 1993). These appear to have coincided with extensive urbanisation and increased opportunity for travel both within and between countries in Africa (Larson, 1989; Anderson *et al*, 1991). Migration across West Africa characterises the demography of the region (Quinn, 1994). Female prostitutes studied in the town of Ziguinchor, Senegal were mainly of Guinea Bissan nationality and, compatible with other epidemiological findings regarding HIV-2 infection in that country, were of a significantly older age-group (Kanki *et al*, 1992). Archetypal forms of HIV-2 may be present in human populations which are better tolerated than others although this remains to be substantiated. The movement of individuals infected with HIV-2 in rural areas to a wider population may have been an important factor in the development of the HIV-2 epidemic in West Africa.

Cross-species transmission events may therefore have occured over time from hosts where these exogenous viruses have persisted but have not caused disease, to a distantly related host such as man where the virus may cause extensive pathology. What is particularly interesting and an apparent paradox is the lack of a convincing candidate giving rise to HIV-1 zoonotic events in view of the impact and spread of HIV-1 worldwide. In contrast, a suitable simian candidate for HIV-2 cross-species transmission has been identified, yet HIV-2 remains largely confined to West Africa and has been associated with reduced virulence. It is the differences from HIV-1, rather than the similarities, that make HIV-2 interesting and therefore comparisons of the biology of the two viruses a key issue (Kanki and De Cock, 1994).

7.5. Differences in pathogenesis between HIV-1 and HIV-2.

Is HIV-2 really less pathogenic than HIV-1? Taking together the most recent epidemiological data from West Africa, from studies based in Senegal (Marlink *et al*, 1994), The Gambia (Whittle *et al*, 1994) and the Ivory Coast (De Cock *et al*, 1993) it would seem that overall patients infected with the HIV-2 group of viruses have a longer survival following infection than similar individuals infected with HIV-1. Epidemiological studies have also shown an increase in the prevalence of HIV-1 in West African countries in recent years compared with HIV-2 which has remained relatively stable in the same populations (De Cock *et al*, 1993). Evidence exists for reduced heterosexual transmission of HIV-2 compared with HIV-1, adding to the view that HIV-2 is both less transmissible and less pathogenic than HIV-1 (Kanki *et al*, 1994). However, members of the HIV-2 group of viruses are unquestionably pathogenic since infection with HIV-2 may result in a clinically indistinguishable disease to HIV-1-related AIDS and is associated with excess mortality. However, it has also become increasingly clear in recent years that the HIV-2 group of viruses vary greatly in their ability to induce disease in the host and that certain HIV-2 strains may be more pathogenic than others. Explanations to account for apparent differences in HIV-2 biology include strain-related virulence (Gao *et al*, 1994) and the lower virus loads in HIV-2-infected individuals (Simon *et al*, 1993). One effect of this has been to keep HIV-2 largely confined to West Africa (DeCock *et al*, 1993).

Viral load has been related to HIV pathogenesis although to date studies relating specifically to HIV-2 remain few, including those described in this thesis, but can be viewed in the context of studies of HIV-1 and animal models using SIV-infected macaques.

In HIV-1 infection it has been demonstrated that during the asymptomatic phase of the disease when the viral burden in the peripheral circulation is low, HIV-1 is actively replicating in the lymph nodes which are sequestering virus and continuing productive infection (Pantaleo *et al*, 1993; Embretson *et al*, 1993). High levels of HIV-1 proviral DNA have also been found in post-mortem samples from patients who had died from AIDS, indicating significant infection of the brain, lung, colon, liver, spleen and lymph nodes as well as the peripheral circulation (Donaldson *et al*, 1994). Using SIVmac251 in rhesus macaques, a high level of viral RNA was demonstrated in the lymph nodes in the early stages of infection which was subsequently transmitted to different target cells (Chakrabarti *et al*, 1994). SIV was demonstrated to replicate in both lymph node macrophages and lymphocytes in primary SIV infection. Follicular dendritic cells were implicated as the major reservoir of virus representing a probable source for dissemination of infectious virus. Individuals infected with HIV-2 have a mild, detectable lymphadenopathy although the long-term role of the lymphoid system in HIV-2 pathogenesis remains unknown. HIV-2 has been detected in the brain tissue of patients with neurological abnormalities using sensitive DNA and RNA PCR techniques, *in situ* hybridisation and immunohistopathology (Dwyer *et al*, 1992). The laboratory findings of this study were consistent with the clinical picture of HIV-2-associated encephalopathy where active viral replication was demonstrated. Further studies of HIV-2-related CNS disease are required.

SIVagm proviral DNA levels have also been measured and found to be similar to those found in asymptomatic HIV-1 infected individuals (Hartung *et al*, 1992). The levels of provirus detected in asymptomatic HIV-2-infected patients where the CD4 count was high, is similar to that in HIV-1 (Chapter 5). Proviral loads have also been compared in both asymptomatic macaques and those with AIDS and found to be approximately 100-fold higher in the latter (Hirsch and Johnson, 1994). Proviral DNA load has been

assessed in lymphoid cells in SIV-infected macaques where both integrated and unintegrated viral DNA was measured (Rosenberg *et al*, 1994). Two SIV isolates (SIVmac251 and SIVmne/E11S) which differed in biological and and clinical properties were used in this study. SIVmac251 induces rapid onset of disease and death while the E11S strain takes up to several (>2) years before symptoms appear. Significant differences were observed between SIV251 and SIVE11S with at least a 10-fold lower level of virus-infected lymph nodes with the latter isolate. In early SIVE11S infection many tissues were found not to contain provirus. Although no strong association between total viral DNA levels and the CD4% in lymphoid tissue was demonstrated, there was a strong correlation between the accumulation of 2-LTR circular unintegrated DNA and a decline in CD4/CD8 ratios which was subsequently related to advanced disease. The presence of unintegrated DNA in lymphoid tissue during late-stage SIV-disease is thought provoking.

In HIV-1 infection, 2-LTR circular species of unintegrated provirus in PBMCs have been detected (Jurrians *et al*, 1992b), although they were detectable only in low amounts. Decreased amounts of unintegrated DNA have also been found in patients receiving zidovudine chemotherapy with the highest levels of unintegrated DNA found in AIDS patients not receiving treatment (Dickover *et al*, 1992). The observed reduction was attributed to the anti-retroviral effects of zidovudine, reducing the overall level of virus replication. This would perhaps support the generally held view that admistration of clinically efficacious anti-retroviral chemotherapy will result in the short-term benefit of reducing viral load, which will ultimately translate into longer term survival of HIV-infected individuals. The use of suitable and comparable anti-retroviral therapy for the treatment of HIV-2-infected individuals remains in the future.

Immunological mechanisms may also be more efficient at controlling HIV-2 infections compared with HIV-1. Differences in cytokine responses, such as tumour necrosis factor, between HIV-1 and HIV-2 have already been discussed and may interact with other aspects of the immune response to contribute to the control of HIV-2 infection. Lower levels of programmed cell death (apoptosis) in HIV-2-infected individuals compared with HIV-1-infected at similar stages of disease have been described (Jaleco *et*

al, 1994). A strong autologous neutralising antibody response has also been demonstrated in nine HIV-2-infected patients (Bjorling *et al*, 1993). This contrasts with the majority of HIV-1-infected patients which appear to lack such antibodies. Furthermore, a strong and specific MHC-restricted cytotoxic T-lymphocyte response in HIV-2 infection may play an important role in maintaining the level of HIV-2 suppression (Gotch *et al*, 1993). Further data from the MRC unit in Fajara comparing the CTL response in HIV-2-infected subjects with proviral DNA, measured using the quantitative assay described in this thesis, have shown an inverse correlation between these two variables (Ariyoshi *et al*, 1995). Strong CTL responses were mounted to at least two structural gene products (*gag* and *pol*) and to a lesser degree to one accessory protein (*nef*). Low cellular HIV-2 DNA loads were therefore associated with a strong CTL response. Interestingly, recent published findings from The Gambia have shown that some female prostitutes may remain uninfected with HIV-2 (or HIV-1) in spite of being repeatedly exposed to HIV-infected males over a period of up to five years (Rowland-Jones *et al*, 1995). A study of HIV-1-infected women in Nairobi also indicates that a small proportion may also be genuinely resistant to infection (Plummer *et al*, 1993).

Taking these observations together, an altered cytokine response, a strong autologous neutralising antibody response and an effective CTL response may act in concert to make the immune response to HIV-2 infection more effective than for HIV-1. These reports are few but provide possible clues as to why HIV-2 infection generally has a slower time-course than HIV-1 before pathogenic effects become realised. It may be that HIV-2 has been present in humans for much longer than HIV-1 and differing pathogenic variants have been tempered by, or resulted in, a generally more effective immune response to infection with HIV-2, further ameliorating the induction of disease.

The dynamics of HIV-1 replication and its relationship to pathogenesis are now gradually becoming clearer. The effects of HIV-1 replication have been clearly linked to the loss of CD4+ lymphocytes, and to rapid and continuous turnover of virus and CD4+ cells *in vivo* (Ho *et al*, 1995; Wei *et al*, 1995). Measurement of replication-competent virus in conjunction with changes in other parameters associated with HIV-disease are therefore providing further insight into the mechanisms of pathogenesis. Increased viral replication

of HIV-1 correlates with increased cytopathy, the fall in CD4 cell numbers and the onset of clinically recognised symptoms (Connor *et al*, 1993) and HIV-1 plasma viraemia levels have been demonstrated to be high at all stages of infection, including the asymptomatic phase (Piatek *et al*, 1993). The slower progression to AIDS in HIV-2-infected patients may be related to both lower levels of plasma viraemia as well as, and possibly as a reflection of, profound differences in the pathogenic properties of HIV-2 strains. If epidemiological and biological differences between HIV-1 and HIV-2 infections are either the cause of, or an explanation for differences in viral load, intervention strategies which focus on this may yet prove to be an effective strategy for controlling HIV disease. The accumulation of provirus in the cells of the peripheral circulation appears to correlate with CD4+ decline yet the temporal expression, level of production of actively replicating virus and viral dynamics of HIV-2 remain to be determined. A lower level of expression of HIV-2 *in vivo*, perhaps as result of different transactivation mechanisms, may result in HIV-2 having a milder effect in the early stages of infection which may in turn allow control to be effected by immune surveillance and HIV-2 better tolerated by its host, reflected in a longer asymptomatic state.

Differences in the perinatal transmission rates (Adjorolo-Johnson *et al*, 1994) may be the most graphic reflection of the differences in pathogenicity and transmission between HIV-1 and HIV-2. Understanding the differences in biology between HIV-1 and HIV-2 remains important in furthering our understanding of the underlying mechansims involved in AIDS pathogenesis and for the development of intervention strategies as part of a measured approach to the treatment of HIV disease and AIDS in general.

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Appendix 1.
Common buffer formulae.

1XTEN
0.1M NaCl
10mM Tris-HCl (pH 8)
1mM EDTA

TE buffer
10mM Tris-HCl (pH 7.6)
1mM EDTA

Conjugate diluent
2% Saponin
10mM EDTA

TMB
0.15M Trisodium citrate
0.02% hydrogen peroxide

Complete RPMI 1640
500ml bottle contains:
$5X10^4$ units penicillin-streptamycin
5 mg/ml kanamycin
200mM L-glutamine
3 or 10% FCS (maintenance/growth)

Tween-saline wash
5 litres of 10X stock:
425g NaCl
25mls Tween-20
0.05% Bronidox

DNA lysis buffer
10mM Tris-HCl (pH8)
10mM EDTA
10mM NaCl
0.5% SDS
100µg/ml proteinase K

Freezing medium
10% DMSO
40% FCS
50% RPMI

Rapid DNA extraction buffer
50mM KCl
10mM Tris-HCl (pH 8.3)
2.5mM $MgCl_2$
0.45% Nonidet P-40
0.45% Tween 20
150µg/ml proteinase K

TY-medium 1 litre:
16g bactotryptone
10g bacto-yeast extract
5g NaCl
Adjust to pH7 with 5N NaOH

Tris-borate buffer (5X TBE)
54g Tris-base
27.5g boric acid
20mls 0.5M EDTA (pH8)

Tris-acetate-EDTA (50XTAE)
1 litre 50X stock:
242g Tris base
57.1 ml glacial acetic acid
100ml 0.5M EDTA (pH8)

RNAzol (Stock A)
23.6g guanidinium thiocyanate
1.25mls 1M sodium citrate
0.5g sarcosyl
make up to 50mls with distilled water

2X Bind and wash buffer
10mM Tris-HCl (pH 7.5)
1mM EDTA
2M NaCl

Appendix 2.
Supplementary data.

Appendix 2.

BG 79 (µg/ml)	Serum dilution	Antigen dilution 1/1000	1/2000	1/4000	1/8000
10	50	224	134	82	55
	400	1086	515	257	149
	3000	2236	1366	758	384
	9000	2359	1823	1054	625
	NHS	2486	1943	1266	702
5	50	139	129	97	65
	400	711	619	371	211
	3000	1979	1702	1242	705
	9000	2257	2163	1744	1204
	NHS	2381	2295	2036	1458
2.5	50	245	140	89	60
	400	1201	570	297	172
	3000	2335	1638	935	458
	9000	2438	1916	1187	729
	NHS	2495	2206	1539	873
1.25	50	61	56	54	46
	400	209	191	167	148
	3000	709	671	539	526
	9000	1163	1027	903	858
	NHS	1282	1187	1129	1034

Table 1. Optical densities in a cross-titration experiment for optimisation of the HIV-2 recombinant competitive EIA. Differing concentrations of BG-79 capture antibody were used to capture antigen at dilutions of 1/1000, 1/2000, 1/4000 and 1/8000 and evaluations made with DJ serum diluted in NHS at 1/50, 1/400, 1/3000 and 1/9000 dilutions.

	Monotypic assays			Combination assays			
Sample	VC-EIA	RC-EIA	ELAVIA-2	Wellcozyme	Abbott	ELAVIA	Behring
Ma S	2300	2000	3500	3100	250	520	700
DN	9000	3400	3750	6000	500	800	3000
BD 49	700	200	120	25	20	15	20
FS	4600	3200	1500	3200	200	900	370
KT	5200	1500	2000	1500	500	600	700
MS	2600	3800	1200	300	250	375	2000
SS	1150	4000	3000	2200	400	450	500
DJ	7500	2000	3200	2000	400	700	1200
BD 568	3300	400	1700	2000	250	350	1600
T493	1600	850	700	600	150	190	600
T347	250	N.T.	100	25	40	20	N.T.
T199	6000	N.T.	2500	5500	350	700	2000
T289	3500	1300	1200	1200	500	375	300
BM	5600	4600	1600	1000	200	450	350
B-25	2100	400	1200	9000	100	600	400

Table 2. Interpolated end-points of 15 HIV-2 sera tested for anti-HIV-2 reactivity in monotypic and combination assays. NT=not tested.

966																										1043
HIV-2ROD	GGC	GGC	AAC	TAC	ACC	CAT	ATA	CCG	CTG	AGT	CCC	CGA	ACC	CTA	AAT	GCC	TGG	GTA	AAA	TTA	GTA	GAG	GAA	AAA	AAG	TTC
CBL-20	---	---	---	--T	---	---	G-G	--A	--A	---	---	---	---	--G	---	--T	---	---	--G	---	---	---	--G	---	---	---
CBL-22	---	---	---	--T	---	---	G-G	---	--A	---	---	---	---	--G	---	--T	---	---	--G	---	---	---	--G	---	---	---
CBL-21	---	---	---	--T	---	---	G-G	---	---	---	---	---	---	---	---	--T	---	---	--G	---	---	---	---	--G	---	---
CBL-23	---	---	---	--T	---	---	G-G	---	---	---	--T	---	---	---	---	--T	---	---	---	---	---	---	---	--G	---	---

1044																										1121
HIV-2ROD	GGG	GCA	GAA	GTA	GTG	CCA	GGA	TTT	CAG	GCA	CTC	TCA	GAA	GGC	TGC	ACG	CCC	TAT	GAT	ATC	AAC	CAA	ATG	CTT	AAT	TGT
CBL-20	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--G	--T	--T	--G	---	---	---	---
CBL-22	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--A	--T	--G	---	---	---	---
CBL-21	---	---	--G	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--C	---
CBL-23	---	---	--G	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

1122																			1178
HIV-2ROD	GTG	GGC	GAC	CAT	CAA	GCA	GCC	ATG	CAG	ATA	ATC	AGG	GAG	ATT	ATC	AAT	GAG	GAA	GCA
CBL-20	---	---	---	---	---	---	--T	---	--A	---	---	---	---	--C	--T	---	--A	---	---
CBL-22	---	---	---	---	---	---	--T	---	--A	---	---	---	---	--C	-CT	---	C-A	---	---
CBL-21	---	---	---	---	---	---	--T	---	--A	---	---	---	---	--C	--T	---	--A	---	---
CBL-23	---	---	---	--	--	---	--T	---	--A	---	---	---	---	--C	--T	---	--A	---	---

Table 3. Nucleotide sequence of CBL.20-23 p26 (bases 966-1178). Sequences numbered according to LAV-2_{ROD}. Accession number M15390.

	1512																									1589
HIV-2ROD	**CTG**	**CTA**	**GTA**	**CAA**	**AAT**	**GCC**	**AAC**	**CCA**	**GAC**	**TGT**	**AAA**	**TTA**	**GTG**	**CTA**	**AAA**	**GGA**	**CTA**	**GGG**	**ATG**	**AAC**	**CCT**	**ACC**	**TTA**	**GAA**	**GAG**	**ATG**
CBL-20	--A	---	---	--G	---	---	---	---	---	--C	--G	---	--A	---	---	---	--G	---	---	--T	--C	---	C--	---	--A	---
CBL-22	---	---	A--	--G	---	---	---	---	---	---	--G	---	--A	---	---	---	---	---	---	--T	---	---	---	---	---	---
CBL-21	---	---	---	--G	---	---	---	---	---	--N	--G	---	--A	---	---	---	---	---	---	--T	---	-G-	---	---	---	---
CBL-23	--A	---	---	--G	---	---	---	---	---	---	---	---	--A	---	---	---	---	---	---	--T	---	---	---	---	---	---

	1590																							1661
HIV-2ROD	**CTG**	**ACC**	**GCC**	**TGT**	**CAG**	**GGG**	**GTA**	**GGT**	**GGG**	**CCA**	**GGC**	**CAG**	**AAA**	**GCT**	**AGA**	**TTA**	**ATG**	**GCA**	**GAG**	**GCC**	**CTG**	**AAA**	**GAG**	**GTC**
CBL-20	---	--T	---	--C	---	---	---	--C	--A	---	---	--A	---	--C	---	C--	---	--T	--A	---	--A	---	---	-C-
CBL-22	---	---	---	---	---	---	--G	---	--A	---	---	---	--G	--A	CAC	---	---	---	---	---	T-A	---	---	-C-
CBL-21	--A	--T	---	--C	T--	--A	---	---	--A	---	---	---	---	--C	---	---	---	---	--A	---	T-A	--G	---	-C-
CBL-23	--A	---	---	--C	--A	---	---	---	---	---	---	---	---	---	---	C--	---	---	--A	---	T-A	---	---	-C-

Table 4. Nucleotide sequence of CBL.20-23 p26 (bases 1512-1661). Sequences numbered according to LAV-2_{ROD}. Accession number M15390.

	Pfu A		*Pfu* B		*Taq*	
pROD10 DNA Copies/10µl	Duplicate	Mean	Duplicate	Mean	Duplicate	Mean
1798	51236 41405	46320	22352 16522	19437	3623 3038	3330
352	28273 19432	23852	11245 8299	9772	2746 2422	2584
34	4711 6482	5596	2126 1929	2027	752 985	868
14	2677 3303	2990	556 991	773	318 469	393
3	754 810	782	309 267	288	206 150	178
0	77, 81 87, 79	82	74, 94 77, 86	84	93, 86 87, 91	89

Table 5. Comparison of *Pfu* and *Taq* DNA polymerases at two primer concentrations. *Pfu* A and *Pfu* B represent amplifications with 10 picomoles and 25 picomoles of biotinylated primer respectively using pROD10 DNA diluted in c8166 negative DNA. *Taq* was compared with *Pfu* using10 picomoles of primer only.

Sample	Frequency of negative reactions at reciprocal dilutions of 1	5	25	125	625	3125	Proviral DNA molecules/µl	Proviral DNA molecules/10µl (10^5 PBMCs)
5	1/4	3/4	4/4	-	-	-	1.4	14
9	0/4	1/4	2/4	4/4	-	-	12.1	121
12	0/4	0/4	1/4	2/4	4/4	-	52.0	520
16	0/4	0/4	1/4	3/4	4/4	-	35.2	352
19	0/4	2/4	3/4	4/4	-	-	5.2	52
23	0/4	0/4	0/4	2/4	2/4	4/4	258.9	2589
28	0/4	0/4	0/4	2/4	3/4	4/4	93.5	935
42	2/4	4/4	-	-	-	-	0.7	7

Table 6. Range of HIV-2 proviral DNA levels in HIV-2-infected individuals determined by limiting dilution analysis. One microlitre of DNA input corresponds to 60 nanograms of cellular DNA.

0/4 and 4/4 are Null information

Appendix 3.

Publications arising from collaborative studies.

Pepin, J., Dunn, D., Gaye, I., Alonso, P., Egboga, A., Tedder, R., Piot, P., **Berry, N.J.,** Schellenberg, D., Whittle, H. and Wilkins, A. (1991). HIV-2 infection among prostitutes working in The Gambia: association with serological evidence of genital ulcer diseases and with generalised lymphadenopathy. *AIDS.* **5:** 69-75.

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