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9. Within the thymuses of *Ctla-4*$^{-/-}$ mice, both CD4 and CD8 single-positive populations were increased fourfold. The proportion of double-positive cells was reduced, resulting in a smaller thymus. The expression of the maturation markers CD69, CD5, and CD44 was similar between *Ctla-4*$^{+/+}$, *Ctla-4*$^{+/-}$, and *Ctla-4*$^{-/-}$ mice. For immunofluorescence staining, see (*10*).
10. Increased mean staining intensity of markers on *Ctla-4*$^{-/-}$ αβ T cells in lymph node and spleen included CD5 (threefold), CD28 (two- to threefold), and CD69 (eightfold). The CD4$^+$/CD8$^+$ ratio remained 3/1 in *Ctla-4*$^{-/-}$ mice, and both CD4$^+$ and CD8$^+$ populations showed up-regulation of CD25, CD44, and CD69. For staining, cells were purified and stained as described [J. Penninger *et al.*, *Science* **260**, 358 (1993)]. For detection of specific TCR V_β and V_α expression, lymph node T cells (from two individual *Ctla-4*$^{-/-}$ mice and four to five pooled *Ctla-4*$^{+/+}$ or *Ctla-4*$^{+/-}$ 4-week-old littermates) were double-stained with anti–Thy-1.2 [phycoerythrin (PE) or fluorescein isothiocyanate (FITC)] and anti-TCR V_β6 (FITC), anti-V_β8 (PE), anti-V_β14 (FITC), or anti-V_α2 (PE). All antibodies were purchased from PharMingen.
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13. The double-positive populations increased for CD5 (threefold) and Fas (fivefold) B220-positive cells. The mean staining intensity of CD5 increased two- to threefold and of Fas increased fivefold on B220-positive cells.
14. Spontanous proliferation of *Ctla-4*$^{-/-}$ T cells decreased to 2000 cpm at 24 hours and to background levels at 48 hours. [^{3}H]Thymidine incorporation in lymph node–derived T cells from a 10-day-old *Ctla-4*$^{-/-}$ mouse was 1300 cpm (12 hours) and background levels at 24 hours.
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17. T cells were stimulated for 3 days (with 1 μg/ml of anti-CD3 and 20 U/ml of IL-2) and washed, and 4×10^6 viable cells were further cultured in media without mitogenic stimuli. The number of viable cells was then determined by Trypan blue staining after 24 and 48 hours of culture. Cell numbers after 24 hours were as follows: 1.6×10^6 for *Ctla-4*$^{+/+}$ and 1.5×10^6 for *Ctla-4*$^{-/-}$ T cells; after 48 hours: 0.5×10^6 for *Ctla-4*$^{+/+}$ and 0.6×10^6 for *Ctla-4*$^{-/-}$ T cells.
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25. T cells isolated from 21-day-old *Ctla-4*$^{+/+}$ or *Ctla-4*$^{-/-}$ littermates were cultured in 10-cm plates coated with hamster antibody to mouse CD3ε. Con A supernatant was added after 24 hours. Cells on day 4 were stained with anti-TCRαβ (FITC-labeled) and anti–CTLA-4 (PE-labeled) or antibodies to CD25, CD44, or CD69 (all biotinylated) (*10*).
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Genomic Structure of an Attenuated Quasi Species of HIV-1 from a Blood Transfusion Donor and Recipients

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A blood donor infected with human immunodeficiency virus–type 1 (HIV-1) and a cohort of six blood or blood product recipients infected from this donor remain free of HIV-1–related disease with stable and normal CD4 lymphocyte counts 10 to 14 years after infection. HIV-1 sequences from either virus isolates or patient peripheral blood mononuclear cells had similar deletions in the *nef* gene and in the region of overlap of *nef* and the U3 region of the long terminal repeat (LTR). Full-length sequencing of one isolate genome and amplification of selected HIV-1 genome regions from other cohort members revealed no other abnormalities of obvious functional significance. These data show that survival after HIV infection can be determined by the HIV genome and support the importance of *nef* or the U3 region of the LTR in determining the pathogenicity of HIV-1.

Among people infected with HIV-1 there are some who, even after infection for 10 years or more, remain healthy with no signs of clinical progression to acquired immunodeficiency syndrome (AIDS) and have stable, normal CD4 lymphocyte counts (*1*–*5*). The explanation for the benign course of HIV-1 infection in such long-term nonprogressors (LTNPs) may be stochastic or related to host or viral factors or a combination of both (*6*).

In 1989, a review of the registry of individuals in New South Wales with blood transfusion–transmitted HIV infection revealed that 6 years after transfusion two infected recipients and a common donor were asymptomatic, with normal CD4 counts. A total of seven HIV-1–infected recipients of HIV-1–infected blood from the same donor (D36, a sexually active homosexual male who became infected between December 1980 and April 1981) have been found (Table 1) (*1*, *7*) among recipients of components of units donated by D36 between 3 February 1981 and 24 July 1984 (*8*). The donor and recipients (hereafter referred to as the

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Sydney Bloodbank cohort) have had CD4 lymphocyte counts >500 per microliter of blood (Table 1) that have remained stable for 10.75 to 14 years (9). No cohort member has developed any AIDS-defining condition or HIV-related symptoms or received any antiretroviral chemotherapy. The cohort members are unrelated, and human lymphocyte antigen (HLA) typing failed to reveal any common genetic background (7). Two recipients of blood products from D36 subsequent to April 1981 remain HIV-1–negative. One received triple-washed erythrocytes in March 1984 (10), and the other recipient received erythrocytes in July 1981 (9). One HIV-1–infected recipient, C83, had severe systemic lupus erythematosus (SLE) and a preexisting immunodeficiency related to her underlying disease and drug therapy (11). She died from complications of SLE and immunosuppression in April 1987 (12).

The time to progression to an AIDS-defining illness or to a CD4 count <200 cells per microliter of blood (13) for infected recipients of blood from D36 (excluding C83) was compared with that of 122 patients on the Sydney Bloodbank registry, including pediatric cases, who had transfusion-acquired HIV infection (median time to progression = 6.5 years); the difference was highly significant (exact log rank test 11.8, $P < 0.0001$) (14). Further analyses that included the cohort member with SLE (C83) and either included or excluded pediatric transfusion recipients [who progress more rapidly than adults (15)] in the control group also showed highly significant differences ($P < 0.0001$). The survival of the individuals infected by blood from D36 [six of seven or 85.7% alive for a mean of 12 (10.75 to 14) years after infection] is also markedly better than that of other reported HIV-1–infected transfusion recipients (25% survival at 12 years) (16) or of cohorts of individuals acquiring HIV-1 infection either sexually (≤50% survival at 10 years after infection) (17) or through intravenous drug use (progression rate of ~5% per year) (18).

The amount of HIV-1 DNA in peripheral blood mononuclear cells (PBMCs) of known CD4 cell count from seven of the eight Sydney Bloodbank cohort members (19) was determined by a quantitative DNA polymerase chain reaction (PCR) technique (20) with the primers SK 38 and SK 39 (21). The amount of HIV-1 DNA in the cohort members was very low [as is characteristic of LTNPs (2, 3)] and ranged from <10 to 400 copies of HIV-1 DNA per 10^5 CD4 cells (Table 1). In comparison, a small control group of time-matched transfusion-infected subjects from the Sydney Bloodbank registry had 400 to 4000 copies of HIV-1 DNA per 10^5 CD4 cells, whereas another group of HIV-1–infected individuals with 400 to 650 CD4 cells per microliter of blood also had up to 400 copies of HIV-1 DNA per 10^5 CD4 cells (Table 1). Members of the Sydney Bloodbank cohort, therefore, had peripheral blood CD4 cell amounts of HIV-1 DNA in the same range as other LTNPs (2, 3) and as little as 0.1% that of patients with advanced HIV infection (Table 1).

Table 1. CD4 cell count and HIV DNA copy number in peripheral blood mononuclear cells of the Sydney Bloodbank cohort and control groups. Two members of the cohort have died from causes unrelated to HIV-1: C83 from SLE in April 1987 and C124 from metastatic gastric cancer in December 1994. C54 had a splenectomy in August 1984. The CD4 count is given as cells per microliter of blood. Dates are given as day/month/year. ND, not done (only available DNA was from a sample of unknown CD4 cell count).

Participants	Year of birth	Data infected by transfusion	Latest CD4 count (cells/μl)	HIV-1 DNA (copies per 10^5 CD4 cells)
		Recipients of D36 blood		
D36	1958	–	587	≤400
C18	1912	31/8/83	850	≤300
C49	1954	11/6/84	918	<10
C54	1928	24/7/84	2026	≤100
C64	1926	4/5/83	861	<10
C83	1964	30/12/82	ND	ND
C98	1937	2/1/82	740	≤25
C124	1918	29/4/81	720	<10
		*HIV-infected controls**		
Group 1 (n = 5)	–	5/81 to 6/84	20–430	400–4000
Group 2 (n = 5)	–	–	<99	250–15000
Group 3 (n = 7)	–	–	100–399	90–9000
Group 4 (n = 4)	–	–	400–650	<40–400

*Members of group 1 are progressors from Sydney Bloodbank registry, matched for time since HIV-infected transfusion. Two died from AIDS-related causes (October 1994 and December 1994). Members of groups 2 through 4 are patients distinct from group 1, with progressive disease and grouped by CD4 cell count.

HIV was isolated by conventional techniques from recipient C18, as previously described (1). HIV was also isolated by unconventional culture techniques, including ultraviolet irradiation (22), from recipients C98 and C54 on at least two occasions, each in two separate laboratories. Relative virus replication of PBMC isolates HIV-1_{C18MBC}, HIV-1_{C54}, and HIV-1_{C98} (from recipients C18, C54, and C98, respectively) was compared with the replication of reference isolates HIV-1_{NL43} and HIV-1_{ADA}. All three cohort isolates were replication-competent, though HIV-1_{C98} and HIV-1_{C54} replicated very poorly compared with HIV-1_{C18MBC}, which in turn showed a prolonged replication time relative to the reference isolates (23). All cohort isolates were nonsyncytium-inducing and macrophage tropic (24).

A region of HIV-1 DNA, including the whole of the *nef* gene and U3 region of the long terminal repeat (LTR), was amplified by double- or triple-nested PCR from viral isolates from recipients C18, C54, and C98 as well as from HIV-1 DNA extracted directly from PBMCs of the donor (D36) and recipients C18, C54, and C98 (25). Viral isolates were obtained from the same samples of PBMCs that were used for PBMC DNA preparations. The resulting amplified fragments ranged in size from ~410 base pairs (bp) for isolate HIV-1_{C18MBC} to ~680 bp for isolate HIV-1_{C98} compared with the ~840-bp fragment from the molecular clone pNL4-3 (26, 27) and from the PBMCs of two noncohort patients infected by transfusion (C53, a nonprogressor, and C59, a progressor) of blood from other HIV-1–infected donors (Fig. 1). This range of fragment sizes indicates a deletion of ~160 to 430 bp from

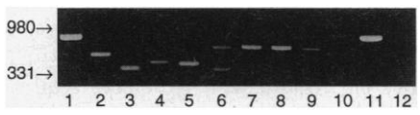

Fig. 1. Analysis of cohort HIV-1 *nef* gene–LTR PCR amplified fragments. DNA products of the third round of amplification were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. The amplified fragments were from DNA from molecular clone pNL4-3 (lane 1); PBMCs from D36 (lane 2), C18 (lane 4), C54 (lane 6), and C98 (lane 8); HIV-1 isolated from C18 (lane 3) and C98 (lane 7) PBMCs; and HIV-1 isolated from C18 (lane 5) and C98 (lane 9) macrophages. Blood samples were obtained from cohort members between 29 July 1993 and 7 November 1994. Controls included a no-DNA reagent control (lane 12) and PBMCs of noncohort patients C53 (blood sampled on 18 May 1994), an HIV-positive nonprogressor with 560 CD4 cells per microliter (lane 10), and C59 (blood sampled on 26 May 1994), an HIV-positive progressor with 108 CD4 cells per microliter (lane 11). Migration of molecular size standards is shown on the left in base pairs.

the *nef*-LTR region of the cohort HIV-1 strain. The amplified fragment from the donor (D36) was ~550 bp in length, indicating a deletion of ~290 bp. Recipient C54 PBMCs yielded fragments of two sizes, ~410 and 680 bp (named C54 PBMC-1 and -2, respectively). Isolate HIV-1_{C54-2} DNA produced the larger fragment only (*28*). We have been unable to amplify and sequence the HIV-1 *nef*-LTR region from PBMCs from C49, C64, C83, and C124, although we have been able to amplify single copy cellular genes from the same DNA (*28*). This finding is consistent with the quantitative PCR data for C49, C64, and C124 and, in the case of C83 (the patient who died from complications of SLE and immunosuppression), is incompatible with death due to advanced HIV-1 infection or AIDS and congruent with the clinical findings suggesting fatal SLE.

Sequencing (*29*) of the amplified fragments from the *nef* gene–LTR region (*30*), including that from isolate HIV-1_{C54-2}, indicated three common features (Fig. 2): (i) a deletion of various lengths from the portion of the *nef* gene that does not overlap with the U3 LTR region (*nef*-alone region), (ii) a deletion from at least one part of the *nef*-LTR overlap region, and (iii) duplication and rearrangement of nuclear factor kappa B (NF-κB) and Sp1 sequences upstream of the basal promoter (Sp1) region of the LTR. Although the sizes of the deletions and the duplication region vary, their locations are conserved. The deletion in the *nef*-alone region in C98 sequences is only 16 nucleotides long and is 3′ to, and distinct from, the deletions in the *nef*-alone region of the other isolates and PBMC-derived sequences, including that of the donor (D36). Furthermore, the sequence from the HIV-1_{C54-2} amplified fragment completely lacks the upstream deletion from the *nef*-LTR overlap region (Fig. 2). The rearranged U3 region from the different PBMC- and isolate-derived sequences, although always present, varies in the spacing and number of duplications of NF-κB and Sp1 sites. All sequences, except that of HIV-1_{C54-2}, lacked the splice acceptor 12 (*SA12*) sequence (base pairs 9160 and 9161 of NL4-3), which is located within the upstream *nef*-LTR overlap deletion region (*31*).

Thus, the only deletion common to all sequences is in the downstream *nef*-LTR overlap region, which includes sequence encoding the conserved amino acid sequence block D of Nef (*32*). This is consistent with our detailed studies of antibodies to Nef in all cohort members (except C83) where, despite recognition of full-length Nef, no antibodies recognized the region of Nef encoded by the equivalent of HIV-1_{NL43} nucleotides 9271 to 9317 (*33*). As recipient C98 was infected 2 years before C54 (January 1982 versus July 1984), these data together suggest that the original virus strain transmitted from D36 may have contained only the downstream *nef*-LTR overlap region deletion and a U3 region rearrangement or duplication, and that the upstream deletions in the *nef*-alone region arose after infection.

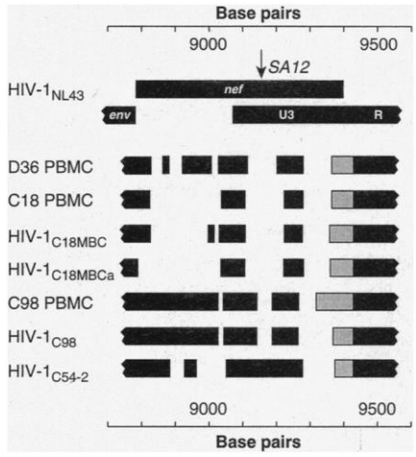

Fig. 2. Sequence abnormalities of the *nef* gene–LTR region of HIV-1 quasi species from the Sydney Bloodbank cohort. Normal sequence is shown by black boxes, the region of NF-κB–Sp1 duplication and rearrangement is shown by light shading, and blank areas denote deletions. The genomic structure of HIV-1_{NL43} for the same region is shown at the top together with a scale of its sequence numbering (*27*) and the site of splice acceptor 12 (*SA12*) (*31*).

Full-length HIV-1 DNA of replication-competent isolate HIV-1_{C18MBC} was amplified by PCR in overlapping fragments (*34*), which were cloned and fully sequenced (*29*). The only obvious differences between the sequence of isolate HIV-1_{C18MBC} (*30*) and that of reference HIV-1 strains (for example, HIV-1_{NL43}) are in the *nef* gene and U3 region of the LTR (Fig. 2). The encoded Nef protein is truncated from 206 to 24 amino acids in length, due to deletions of 177 and 11 bp from the *nef*-alone region that bring into phase a termination codon at the resulting 25th codon. Downstream there is further loss of *nef* sequences by deletions of 120 and 86 bp from the *nef*-LTR overlap region (Fig. 2). The encoded Nef is identical to the NH_2-terminus of the HIV-1_{NL43} Nef sequence at 9 of the first 10 amino acids; thereafter, homology is lost completely (*35*).

The loss of sequence from the *nef*-LTR overlap region is reflected in the loss of the same sequence from the U3 region of the 5′ LTR. The deletions of 120 and 86 bp, therefore, eliminate a number of binding sites for important HIV-1 transcription factors including the negative regulatory element and sites for NF-AT, NRT-1, USF, and TCF-1α. Furthermore, the sequence between the 3′-most U3 deletion and the normal 3′ NF-κB site has low homology (20%) with the same region of HIV-1_{NL43}. This sequence represents a rearranged duplication of NF-κB–Sp1 enhancer-promoter sequences resulting in one additional perfect NF-κB and two putative Sp1 sites that are 57, 45, and 2 bp, respectively, upstream of the normal 3′ NF-κB site together with a 9-bp insertion (one of the extra Sp1 sites) between the normal 5′ and 3′ NF-κB sites.

The causal relation between the infecting strain and the benign clinical course in the cohort examined here is supported by the epidemiological data and is corroborated by the sequence data that show that all members of the cohort harbor quasi species of HIV-1 with major deletions in *nef* and the *nef*-LTR overlap region and a rearranged duplication of U3 sequences. No other obvious differences from the nucleotide sequences of other fully virulent HIV-1 strains were observed, and such deletions have not been observed in virulent strains of HIV-1 (*27*). Other characteristic features of the HIV-1 quasi species from the Sydney Bloodbank cohort members include the positioning of deletions in the *nef*-alone and *nef*-LTR overlap regions and a rearranged duplication of the NF-κB–Sp1 region. All cohort sequences studied have an intact polypurine tract, Sp1 basal promoter region, TATA box, and TAR sequences, features which are all crucial for virus replication. As these features are common to all of the cohort sequences studied and there appear to be no other significant genomic abnormalities, it is reasonable to infer that these *nef* or LTR defects are responsible for the attenuated infection caused by this HIV-1 quasi species and the severely impaired in vitro replication of two of the three viruses isolated, compared with wild-type HIV-1 isolates. These results are consistent with the earlier finding that the simian immunodeficiency virus strain $SIV_{mac239\Delta nef}$ replicated only poorly in macaques and did not cause disease (*36*). Furthermore, in macaques inoculated with $SIV_{mac239\Delta nef}$, additional deletions arose in *nef*-LTR (*37*), and the macaques were protected from challenge by pathogenic SIV (*38*).

Deletions in the *nef*-LTR region, similar to those in the cohort virus strain, have been reported for one of five LTNPs (*5*). Here also a primary deletion present at the time of infection was followed by additional deletions in *nef*-LTR over a period of 10 years. However, the remaining four LTNPs had no deletions in *nef*. In other studies a single LTNP hemophiliac (*4*) and a group of 10 LTNPs (*2*) were found

to have no deletions in *nef*, although two of the individuals were noted to have undefined defects in the NF-κB and Sp1 sites (*2*). These individuals, together with a group of 15 LTNPs (*3*) infected for 8 to more than 15 years and having stable, normal CD4 cell counts, had PBMC viral loads 1/7 to 1/50 those of progressors. HIV-1 was isolated from only 3 of 10 nonprogressors in one study (*2*), and in the other study, virus was isolated from lymph node mononuclear cells of 7 of the 15 nonprogressors but not from plasma (*3*). The lymph nodes of these nonprogressors had significantly fewer of the hyperplastic and none of the involuted changes characteristic of progressors (*3*). In all three studies LTNPs were shown to have strong cellular and humoral immune responses (including neutralizing antibody) against HIV-1.

The strain of HIV-1 that infected the eight members of the Sydney Bloodbank cohort has not caused disease, even in the members affected by the immunosuppressive effects of age, drug therapy, and SLE. This attenuated strain of HIV-1, therefore, could perhaps be the basis for a live attenuated vaccine. These data also indicate the importance of *nef* and the LTR in production of HIV-related disease and suggest that *nef* and LTR function might be promising targets for antiviral chemotherapy.

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22. HIV-1–negative donor PBMCs were stimulated by culture in RPMI-1640 containing 10% (v/v) heat-inactivated fetal calf serum (FCS), 15 mM Hepes, 0.1% (w/v) sodium bicarbonate, penicillin (100 IU/ml), and streptomycin (100 μg/ml) with the addition of phytohemagglutinin (PHA) (10 μg/ml) (Wellcome, Temple Hill Dartford, England) for 72 hours before co-culture. Fresh patient cells (10×10^6) were then co-cultured with the PHA-activated donor PBMCs (10×10^6). Immediately on co-culture, 2×10^6 of the mixed cell population were irradiated with ultraviolet (UV) light (254 nm, 300 μW/cm², 15s), added back to the remaining cells, and cultured for 29 days. After UV treatment cells were resuspended at 1×10^6 in RPMI-1640 containing 10% (v/v) heat-inactivated FCS, 15 mM Hepes, 0.1% (w/v) sodium bicarbonate, penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (25 μg/ml), polybrene (2 μg/ml) (Sigma, St. Louis, MO), hydrocortisone (4 mg/ml) (Sigma), interleukin-2 (20 U/ml) (Boehringer Mannheim, Germany), and antibody to interferon (120 nU/ml) (ICN Biochemicals, Costa Mesa, CA). Cells were maintained by half-medium changes every 3 to 4 days after PHA stimulation, with the addition of fresh stimulated donor PBMCs on days 7, 14, and 21. Virus production was assayed by cell-free reverse transcriptase activity (*39*) or p24 antigen (Abbott Diagnostics).
23. Time (days after infection) to peak virus production [virion-associated reverse transcriptase (RT) activity (*40*)] of the HIV-1 isolates in PHA-activated PBMCs with an input inoculum of 10^4 cpm RT counts per 10^6 cells was HIV-1_{NL43}, day 8; HIV-1_{ADA}, day 10; HIV-1_{C18MBC}, day 8 to 21; HIV-1_{C54}, day 21; and HIV-1_{C98}, >day 28.
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25. For the reaction (50 μl), ~1.0 μg PBMC DNA was subjected to 94°C for 120 s followed by 35 cycles of amplification (94°C for 15 s, 55°C for 15 s, and 72°C for 60 s) then 72°C for 7 min. Amplification product (2 μl) was added to the second-round reaction and cycled as before. The third round of amplification was carried out similarly. Oligonucleotide primers for triple-nested PCR amplification of the *nef*–LTR U3 region, outer pair: SK 68, bp 7786 to 7805 (*21*) and CI-6, TGCTAGAGATTTTCCACAC, bp 9709 to 9691; middle pair: KS-2, AGTGAATAGAGTTAGGCAGG, bp 8326 to 8345 and CI-6; inner pair: Nef-5′ GTAGCTGAGGGGACAGATAG, bp 8678 to 8697 and LTR-3′, AGGCTCAGATCTGGTCTAAC, bp 9559 to 9540. Double-nested PCR used the outer and inner primer pairs. Numbering is according to the sequence of HIV-1_{NL43} (*27*). Products (10 μl) were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide.
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28. A. Solomon and N. J. Deacon, unpublished results.
29. F. Sanger, S. Nicklen, A. R. Coulsen, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5463 (1977); L.-H. Gou and R. Wu, *Nucleic Acids Res.* **10**, 2065 (1982).
30. Sequence accession numbers (GenBank): U37267 to U37271. The sequence of HIV-1_{C18MBC} is 9207 bp long, 502 bp shorter than HIV-1_{NL43} due to 128 bp of insertions and 630 bp of deletions. Of these, 108 and 30 bp of insertions and deletions, respectively, represent differences ranging from 1 to 30 bp in length heterogeneity (*27*). In two cases, where the *env* reading frame is disrupted, the reading frame is corrected by a second mutation within 10 bp. The *gag* differences are in-phase direct repeats of immediately adjacent sequence and were not found in sequences derived from isolates HIV-1_{C98} and HIV-1_{C54}. The envelope gp120 V3 loop region sequence is that of a clade B virus and is consistent with the observed nonsyncytium-inducing, macrophage-tropic phenotype (*41*) of this and the other isolates from the Sydney Bloodbank cohort.
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34. For the reactions (25 μl), approximately 1 μg of infected PBMC DNA was subjected to 94°C for 30 s then amplified by 35 cycles at 94°C for 15 s, 55°C for 60 s, and 72°C for 5 min with Taq polymerase (Boehringer Mannheim, Australia). Amplification product (2 μl) was added to the second-round reaction and amplified under the same conditions. Conditions for primer pairs CI-26/CI-6B and CI-27/CI-6B were 94°C for 30 s then 35 cycles of 94°C for 10 s and 68°C for 10 min with either Ultma (Perkin Elmer) or KlenTaq 1 (Ab Peptides, St. Louis, MO) polymerases. Final products were ligated into pGEM7Zf+ (Promega) for cloning. Primer pairs used to amplify HIV-1 as four overlapping outer fragments were the following: CL-1A, GGAAGGGCTAATTTACTCCCAAAAAAGAC (bp 1 to 30) and CL-14, AATCGTTCTAGCTCCCTGCTTGCCC (bp 920 to 896); CL-11, TCTCTCGACGCAGGACTCGGCTT (bp 682 to 704) and CL-18, CTGTTTTCTGCCAGTTCTAGCTCTGCTTCT; CL-26, CCACACCAGACAAAAAGCATCAGAAAGAACCCCCATTCC (bp 3193 to 3231) and CL-6B, TGCTAGAGATTTTCCACACGGACTAAAATGGTCTGAGGG (bp 9709 to 9671); SK 68, bp 7786 to 7805 (*21*) and CI-6, TGCTAGAGATTTTCCACAC (bp 9709 to 9691). Inner primer pairs were the following: CL-1B, AATCCCGGGTGGAAGGGCTAATTTACTCCC (bp 1 to 21) and CL-13, CCTCTAGACCGCTTAATACTGACGCTCTCGC (bp 818 to 796); CL-12A, TTTCCCGGGCGGCGACTGGTGAGTAC (bp 732 to 751) and CL-17, CCCTCTAGACTTGCCCAATTCAATTTTCCCAC (bp 3354 to 3330); CL-27, CCATCCTGATAAATGGACAGTACAACCCATAGTACTGCC (bp 3251 to 3289) and CL-28, GTGGCCCAAACATTATGTACCTCTGCATCATATGC (bp 6432 to 6398); CL-19, AGCAGGACATAAGAAGGTAGGATCTCTACA (bp 5448 to 5477) and CL-24, GGATCTGTCTCTGTCTCTCTCCACCT (bp 8449 to 8422); KS-2, AGTGAATAGAGTTAGGCAGG (bp 8326 to 8345) and CI-6, TGCTAGAGATTTTCCACAC (bp 9709 to 9691). Numbering is according to the sequence of HIV-1_{NL43} (*27*). Underlined sequences denote restriction enzyme site tail.
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