JOURNAL OF VIROLOGY, June 1999, p. 4866–4881 Vol. 73, No. 6
0022-538X/99/$04.00+0

Definition of the Stage of Host Cell Genetic Restriction of Replication of Human Immunodeficiency Virus Type 1 in Monocytes and Monocyte-Derived Macrophages by Using Twins

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Received 9 November 1998/Accepted 22 February 1999

Using identical (ID) twins, we have previously demonstrated that host cell genes exert a significant impact on productive human immunodeficiency virus (HIV) infection of monocytes and macrophages (J. Chang et al., J. Virol. 70:7792–7803, 1996). Therefore, the stage in the replication cycle at which these host genetic influences act was investigated in a study using 8 pairs of ID twins and 10 pairs of sex- and age-matched unrelated donors (URDs). In the first phase of the study, blood monocytes and monocyte-derived macrophages (MDM) of ID twins and URDs were infected with 15 HIV type 1 strains. Four well-characterized primary isolates and HIV-BaL were then examined in more detail. The host cell genetic effect in MDM was exerted predominantly prior to complete reverse transcription, as the HIV DNA level and p24 antigen levels were concordant ($r = 0.91$, $P = 0.0001$) and similar between the pairs of ID twin pairs ($r = 0.96$, $P = 0.0001$) but discordant between URD pairs ($r = 0.11$, $P = 0.3$) in both phases of the study. To further examine genetic influence on viral entry, we examined the proportion of CCR5 membrane expression on MDM. As expected, there was wide variability in proportion of MDM expressing CCR5 among URDs ($r = 0.58$, $P = 0.2$); however, this variability was significantly reduced between ID twin pairs ($r = 0.81$, $P = 0.01$). Differences in viral entry did not necessarily correlate with CCR5 expression, and only very low levels of CCR5 expression restricted HIV entry and production. In summary, the host cell genetic effect on HIV replication in macrophages appears to be exerted predominantly pre-reverse transcription. Although CCR5 was necessary for infection, other unidentified host genes are likely to limit productive infection.

As in most viral infections, most of the key factors which determine the outcome after exposure of an individual to human immunodeficiency virus (HIV) are yet to be determined. However, both host and viral factors are likely to play a role. These interactions may determine the likelihood of infection or the rate of progression of disease (63).

The viral factors which have been shown to be important in HIV disease progression include genotype, cytopathicity, and coreceptor usage. For example, mutations in the *nef*-long terminal repeat (LTR) region of HIV infection of a cohort of patients infected via blood transfusion and *nef* in simian immunodeficiency virus infection of macaques reduce or eliminate progression to immunosuppression (13, 38, 47, 66). Key sequences in the V3 region also appear to be associated with the development of severe AIDS dementia complex (39, 61). Furthermore, different HIV strains can utilize different chemokine receptors, and coreceptor usage often changes during progression of HIV disease. The viral load in blood has been shown to be highly predictive of disease progression (44). However, while plasma viral load has been shown to be the best prognostic marker of disease progression in patients with HIV infection, plasma viral load is also likely to represent a balance between viral and host factors (30).

Identified host factors include HLA type and chemokine/chemokine receptor polymorphism. Earlier studies of host genetics showed that the HLAB8 DR3 haplotype was consistently linked with more rapid CD4 cell decline and disease progression (23, 35, 64). Although there have been several reports of host HLA linkage with resistance to HIV infection, the results have been inconsistent. Mutations in chemokine receptors and chemokine genes have clearly been shown to influence the likelihood of HIV infection and also the rate of HIV disease progression. There is now good evidence that heterozygotes for CCR5 Δ32, constituting 20% of the population, have a slower rate of disease progression (14, 59, 62). However, individuals who are homozygous for a 32-base deletion in one of the chemokine receptor genes, CCR5 (14, 32, 42, 67), appear to be almost completely protected against infection, reflecting the importance of CCR5 as a coreceptor with CD4 for macrophage-tropic (M-tropic) and dualtropic HIV entry into cells (2, 15, 18). Mutations in other chemokine receptor genes, either coding or regulatory regions, or chemokine genes have also been associated with slower progression to disease and death. These include the CCR2-^{64}I mutation (40, 72), which is in strong linkage disequilibrium with a mutation in the regulatory region of the closely linked CCR5 gene, and a mutation in the regulatory region of the chemokine stroma-derived factor 1 (80), which binds to CXCR4 (5, 55). However, these are unlikely to be the only host factors determining the rate of progression, as there is a continuum in survival after HIV infection ranging from 9 months to over 15 years, suggestive of human polygenic effects.

These findings are supported by recent definition of the role of the chemokine receptors as coreceptors for HIV in T-cell line-tropic (T-tropic) and M-tropic infection (2, 24). CXCR4

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TABLE 1. HLA-DR, CCR5 Δ32 genotypes, and zygosity identity of twin pairs and URDs used in this study

Pair	Twin 1		Twin 2		Twins' zygosity[b]	URD 1		URD2	
	HLA	CCR5 Δ32[a]	HLA	CCR5 Δ32		HLA	CCR5	HLA	CCR5
Twin									
1	KW (12, −)	WT	PW (12, −)	WT	Concordant				
2	JB (0301, 03)	WT	NB (0301, 03)	WT	Concordant				
3	MV (07, 0301)	WT	AV (07, 0301)	WT	Concordant				
4	PG (07, 15)	WT	CG (07, 15)	WT	Concordant				
5	KW (09, 15)	HT	MW (09, 15)	HT	Concordant				
6	KF (12, 15)	WT	NF (12, 15)	WT	Concordant				
7	MG (01, 02)	WT	RG (01, 02)	WT	Concordant				
8	DH (08, 0101/2/4)	HT	BH (04, 0101/2/4)	HT	Discordant				
9	GS (03, 3/6)	WT	DS (03, 11)	WT	Discordant				
10	PG (02, 04)	WT	NG (02, 03)	WT	Discordant				
URD									
1						KA (01, 02)	WT	BD (02, 04)	WT
2						TA (07, 14)	WT	RF (03, 11)	WT
3						AC (0301, 01)	WT	CR (14, 15)	HT
4						JR (07, 17)	WT	MA (01, 14)	WT
5						SD (07, 6)	WT	MS (07, −)	WT
6						RN (02, 12)	WT	AS (07, 12)	WT
7						ST (04, 6)	WT	NS (01, 02)	HT
8						SH (01, 02)	HT	NH (09, −)	WT
9						JR (09, 01)	WT	FL (14, 17)	HT
10						MN (06, −)	WT	JT (02, 7)	WT

[a] WT, wild type for CCR5; HT, heterozygous for CCR5 Δ32.
[b] Zygosity testing was carried out by using microsatellite markers and multiplex PCR (see Materials and Methods).

appears to be the dominant receptor for syncytium-inducing T-tropic HIV strains, whereas CCR5 appears to be the dominant coreceptor for non-syncytium-inducing M-tropic HIV isolates in infection of primary T lymphocytes and macrophages. However, CCR3, CCR2b, STRL33 (Bonzo), GPR15 (Bob), GPR1 (3, 9, 16, 29, 41), and probably other coreceptors are important for some primary strains (31). Therefore, HIV infection of primary T lymphocytes by M-tropic, T-tropic, and dualtropic isolates may be mediated either by CCR5, by CXCR4, or by both (2, 17, 24). CXCR4-utilizing strains often appear during advanced HIV disease, but at other times CCR5-utilizing HIV strains comprise the majority of transmitted strains (70, 76, 83, 84). The viral determinants which influence binding to either of these two major coreceptors are found mainly within the V3 region, as originally predicted from biological studies of tropism (4, 8, 10, 34, 78, 81).

In animal models, the load of virus in the whole animal reflects the effects of host and virus (25, 68). However, host genetic effects in the whole animal are also reflected in the ability of the virus to replicate in the appropriate target cells from that animal cultured in vitro. This finding suggests that host cell genetic effects determine the overall productivity of infection by interactions between virus and cell at various stages of the replication cycle. In vitro evidence for the host genetic influence on HIV disease was first manifested by variation in the ability to culture virus to high concentrations depending on the donor of the cocultured T cells (22). Williams and Cloyd (79) showed up to a 1,000-fold variation in the susceptibility of mononuclear cells from different donors to infection with T-tropic HIV strains. Spira and Ho (74) showed less variation (up to 40-fold) in HIV production from different donor mononuclear cells after infection with primary isolates.

Blood monocytes and tissue macrophages are viral reservoirs and therefore may contribute to disease pathogenesis and the response to antiretrovirus therapy. Recently (7) we published data from a study using monocytes from identical (ID) twins and unrelated donors (URDs) which showed a host cell genetic effect on HIV replication in these cells. The kinetics of HIV production as measured by extracellular (EC) HIV antigen were concordant in all of the ID twins (as defined by prospective criteria) and discordant in 10 of 12 pairs of URDs, a highly significant difference. Therefore, in this study we examined the stage of HIV replication at which host genetic factors influence HIV infection and replication in monocytes/macrophages by comparing pre- and post-reverse transcription (RT) events. Reports from this and other laboratories have clearly shown altered susceptibility to HIV infection depending on the state of maturation of monocytes into macrophages (51). We have also shown that the susceptibility of monocytes and monocyte-derived macrophages (MDM) to infection is increased when isolates of HIV from advanced infection (i.e., AIDS) are used (53a). Therefore, both monocytes adherent for only 16 h and MDM adherent for 3 and 5 days were infected with a range of blood isolates from both early and late stages of HIV infection in these experiments. While we found that host factors primarily influence events prior to complete RT, we found that this did not correlate with levels of CCR5 expression or with CCR5 Δ32 heterozygous status.

MATERIALS AND METHODS

Subjects. Eight pairs of ID and three pairs of non-ID (NID) Caucasian twins aged 35 to 45 years were recruited from the Australian NHMRC Twin Registry, and 10 pairs of approximately age matched URDs (32 to 41 years old) of the same sex were randomly recruited from healthy HIV-seronegative Caucasian staff, consecutively assigned as pairs, and used in this study. The identity of twins was confirmed by phenotype (maternal interview), by HLA-DR genotyping, and by a zygosity assay (Table 1).

HLA genotyping. All ID twin pairs and URDs were typed for HLA at the DR locus by PCR amplification with sequence-specific primers (57). Briefly, genomic DNA was extracted and purified by the salting-out method (48); then the PCR target sequence was amplified by using standard PCR conditions and primers specific for HLA-DR alleles. Amplified products were separated on a 2% agarose gel, and allelic assignments were analyzed as described by Olerup and Zetterquist (57).

TABLE 2. Clinical and virological characterization of HIV-1 primary isolates used in this study

Patient	Stage of disease[a] (category)	CD4 counts[b]	Clinical status	Tissue of origin
1123	II	500	Asymptomatic, HIV positive	Blood
1124	II	200	Asymptomatic, nontransmitting mother	Blood
1127	II	350	Asymptomatic, HIV positive	Blood
1117	IV	<50	AIDS	Blood
1114	II	200	Asymptomatic	Blood
1044	IV	<50	AIDS with encephalopathy	Brain
1097	IV	<50	AIDS, rapid progressor	Blood
1039	II	400	Asymptomatic, nontransmitting mother	Blood
1067	II	450	Asymptomatic, HIV positive	Blood
1068	IV	<50	AIDS, advanced disease	Blood
1076	IV	<50	AIDS	Blood
MW	IV	<50	AIDS rapid progressor with encephalopathy	Blood
333	IV	<50	AIDS, rapid progressor	Blood
933	IV	<50	AIDS	Blood
1192	IV	<50	AIDS	Blood
1101	IV	<50	AIDS	Blood
1052	IV	<50	AIDS	Lung

[a] According to CDC classification (category IV, AIDS; category II, asymptomatic; category I, acute seroconversion).
[b] Absolute count of CD4 lymphocytes per microliter of blood.

Zygosity assay. Confirmation of zygotic identity (as ID or NID) within twin pairs was also examined by typing for polymorphic microsatellite markers. Markers were selected from a number of different chromosomes. Following DNA extraction from peripheral blood mononuclear cells (PBMC) (48), multiplex PCR amplification of DNA was performed for 30 cycles. Eight highly polymorphic DNA microsatellite markers from different chromosomes were chosen to determine zygosity. The DNA was multiloaded and run on a single-Applied Biosystems (ABI) 373 Genescan machine, and genotypes and zygosity were determined by the ABI program Genotyper. The primers used in the five PCRs were D9S265 and TYRP2A (fluorescence-labeled TET; green), FGF3 and D12S356 (fluorescence labeled HEX; yellow), D6S89 and TGFA (fluorescence-labeled 6-FAM; blue), D4S192 (fluorescence-labeled 6-FAM), and D1S214 (fluorescence-labeled HEX).

Viruses. HIV strains were obtained from 17 patients at all stages of HIV infection and disease from asymptomatic to symptomatic with blood CD4 concentrations ranging from 500 to 50 (Table 2). Two groups of freshly isolated HIV type 1 (HIV-1) strains were used in two phases. The first phase included the analysis of replication kinetics of 15 primary HIV-1 isolates cultured in monocytes and MDM from three pairs of ID twins and three pairs of URDs. In the second phase, four primary isolates with different levels of replication in MDM and HIV-BaL were used to infect 3-day-old MDM from another five pairs of ID twins and also 7 age- and sex-matched pairs of URDs. Three-day-old MDM were used in phase II, intermediate between the 1- and 5-day MDM used in phase I. An input multiplicity of infection (MOI) of 0.02/cell was used for phase I; the MOI was increased to 0.1/cell in phase II.

Peripheral blood samples were collected in heparin and processed within 2 to 3 h of phlebotomy. Isolates used in this study were from a single passage of cocultivation with phytohemagglutinin-stimulated PBMC from random heterologous blood donors to produce virus stocks. These isolates were tested for the ability to infect 3-day-adherent MDM. Titers of virus stocks were determined by endpoint dilution in 96-well tissue culture plates with phytohemagglutinin-stimulated PBMC. The 50% tissue culture infective dose ($TCID_{50}$) was determined by measuring the level of the EC HIV p24 antigen in culture supernatants by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Coulter Electronics, Sydney, Australia). p24 antigen levels at their peak at either day 7 or day 14 after HIV infection in monocytes/MDM were classified as undetectable (<25 pg/ml; 0), low (26 to 500 pg/ml; 1+), intermediate (500 to 5,000 pg/ml; 2+), high (5 to 50 ng/ml; 3+), or very high (>50 ng/ml; 4+). Care was taken to batch all of the supernatants for p24 antigen assay from the same experiment in the same run. Assays were performed in triplicate. Approximately equal numbers of monocytes (10^6) were exposed to the same virus inoculum (MOI of 0.02 $TCID_{50}$/cell as propagated in PBMC) of both clinical isolates and HIV-BaL. HIV-BaL (28) was obtained from the NIH AIDS Research and Reference Reagent Program and used as a control M-tropic strain.

Monocyte isolation. Blood-derived monocytes were isolated from 150 ml of whole blood from healthy HIV-seronegative donors as previously described (36). Briefly, PBMC were obtained by differential centrifugation on Ficoll-Hypaque (Pharmacia-AMRAD, Sydney, Australia). Monocytes were separated from PBMC by countercurrent elutriation (Beckman model J-6M/E/centrifuge fitted with a JE 5.0 elutriation rotor) and OKT3/complement lysis as an extra step to purify monocytes from contamination with T cells. The isolated monocyte populations were >96% positive for nonspecific esterase. Cells were cultured in the absence of growth factors in 1.0 ml of 10/10 medium (RPMI 1640 supplemented with antibiotics, 10% heat-inactivated fetal bovine serum, and 10% heat-inactivated pooled AB^+ human serum) at a density of 10^6 per well in a 24-well tissue culture plate (Nunc, Sydney, Australia). Cultures were replenished with fresh 10/10 medium every 3 or 4 days. Monocytes were allowed to adhere for 3 to 5 days to differentiate into macrophages, >99% pure as judged by cell morphology and macrophage surface markers (CD14, CD11b, and CD26). Monocytes and MDM were infected with the same panel of HIV-1 clinical isolates and HIV-BaL after 16 h (data not shown) and 5 days, respectively, of adherence in phase I experiments and after 3 days of adherence in the second phase. Culture supernatants were examined for HIV-1 p24 antigen, and cells were lysed for DNA on designated days. Levels of EC HIV p24 antigen in culture supernatants were determined by a commercial ELISA as instructed by the manufacturer (Coulter). Antigen amounts (in nanograms per milliliter) were calculated, and values greater than 25 pg/ml were considered positive.

DNA extraction and hot PCR amplification. DNA lysates from uninfected and HIV-infected monocytes and MDM were prepared as previously described (52). Briefly, cells were lysed with DNA lysis buffer containing proteinase K at 60°C for 1 to 2 h, then incubated at 95°C for 10 to 15 min, and stored at −20°C until used for PCR. HIV-1 DNA was amplified by PCR using 2.5 U of *Taq* polymerase, a 0.2 mM concentration of each of the four deoxyribonucleoside triphosphates, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.01% gelatin. Primers M667 (82) and *gag*1 (52) were used to amplify a 320-bp region extending from the R region within the 5′ LTR to the beginning of the *gag* region, representing almost full-length synthesis of HIV cDNA. In a subset of samples, primers M667 and AA55 (82) were also used to amplify a 140-bp region flanking the R and U3 regions of the 5′ LTR, representing the initiation product of HIV cDNA synthesis. Samples were subjected to 30 cycles of amplification in a Perkin-Elmer Cetus thermal cycler as follows: 1 min at 95°C, 2 min at 60°C, and 3 min at 72°C, with a final extension at 72°C for 7 min. Concurrent reactions were also performed with primers PCO3 and PCO4 to amplify a 110-bp DNA fragment of the human β-globin gene (65) to ensure that equivalent amounts of DNA were used in all sample reactions. PCR products were electrophoresed on a 2% agarose gel, visualized by using ethidium bromide staining and a UV transilluminator, and then photographed. Hot PCR was carried out by incorporating [γ-^{32}P]ATP into one of the PCR primers (M667) added at a hot-to-cold ratio of 1:2 to the same cold primer in a PCR mix of 50 μl. PCR products were run on 2% agarose gel, dried, and then exposed to X-ray film for 4 to 6 h.

DNA extracted from 8E5 cells, containing one integrated copy of HIV-1 DNA per cell (27), was used to construct a standard curve for quantification of HIV DNA from monocytes and MDM. Tenfold dilutions (0, 10, 10^2, 10^3, and 10^4 cells/reaction) of 8E5 cells were prepared as described above. The differences in cell number between these dilutions was compensated for by addition of uninfected PBMC (to a total of 10^5) as background. HIV DNA levels were classified as 0 (undetectable), 1 (10 to 10^2), 2 (low copy number [10^2 to 10^3]), 3 (intermediate copy number [10^3 to 10^4]), and 4 (high copy number [>10^4]) as normalized to the HIV DNA standard curve. For HIV DNA PCR, parallel cell cultures were treated with zidovudine (20 μM) for 30 min at 37°C to control for de novo HIV DNA synthesis. Treatment with zidovudine reduced HIV-BaL DNA levels from 3+ to undetectable.

CCR5 genotyping, expression, and usage by HIV isolates. CCR5 genotyping for the 32-bp deletion was carried out as previously described by Samson et al. (67). DNA fragments of 183 bp for the wild type and 151 bp for the deletion mutant were visualized on an ethidium bromide-stained agarose gel and photographed.

Flow cytometry was carried out to examine the cell surface expression of CCR5 and CXCR4 in 3-day-old monocytes. After cells were removed from the plastic surface by using 5 mM EDTA in phosphate-buffered saline they were washed twice with cold fluorescence-activated cell sorting buffer containing 1% fetal bovine serum and 0.01% sodium azide in phosphate-buffered saline, then resuspended in 50 μl of human serum, and labeled with specific antibody as previously described (51). Cells were examined in a Becton Dickinson (Franklin Lakes, N.J.) FACScan flow cytometer. Monoclonal antibody 2D7 for CCR5 was obtained from LeukoSite, Inc. (Cambridge, Mass.), and monoclonal antibody 12G5 for CXCR4 (19) was purchased from R & D Systems (Minneapolis, Minn.). Anti-Leu3a-fluorescein isothiocyanate conjugate was purchased from Becton Dickinson.

Coreceptor usage by HIV-1 isolates was examined by using HOS.CD4 cells transfected with CCR1 TO CCR5, CXCR4, Bonzo, and Bob, all kindly donated by D. Littman (New York University Medical Center, New York, N.Y.) (15). HIV infection was determined by PCR on cell lysates for HIV DNA and by p24 antigen ELISA on culture supernatants.

DNA sequencing of V3 and HIV accessory genes. The V3 region and regulatory gene *vpr* genes were amplified from HIV-infected MDM by using nested PCR. The first round of amplification was carried out for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Aliquots (5 μl) of the amplified products were included in the second round of amplification, using specific primers for each gene for 25 cycles using the same conditions as for the first round. The V3

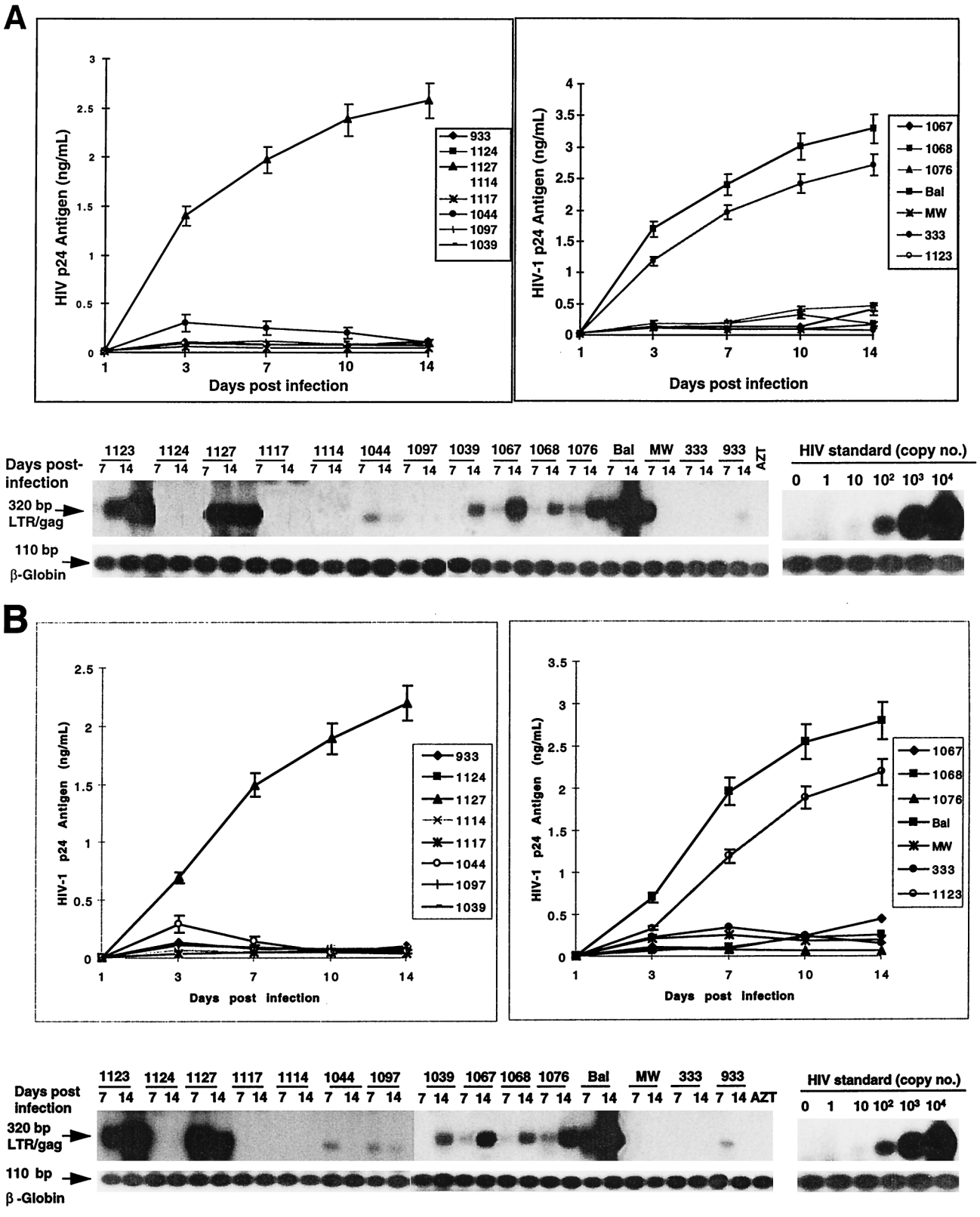


FIG. 1. Phase I: ID twin pairs. Shown are the kinetics of replication of primary HIV isolates in 5-day-old MDM from ID twin pairs (A and B). Cells were infected with 15 different isolates at an MOI of 0.02 $TCID_{50}$/cell. The replication kinetics of isolates was measured as the EC p24 antigen in culture supernatants by using a commercially available ELISA kit (Coulter) and by quantitative PCR for the detection of nearly full length HIV cDNA (320 bp) after RT. Cultured supernatants were collected 0, 3, 7, 14, and 21 days after infection for the detection of HIV p24 antigen. DNA was extracted from cells at 7 and 14 days after infection for detection and quantification of HIV DNA by PCR. β-Globin DNA (110 bp) was amplified concurrently with HIV DNA, and results are shown below each DNA sample. HIV DNA standards were constructed by using 8E5 cells (27). Zidovudine (AZT)-treated cells provided control for de novo HIV RT. Note the marked similarities in kinetics of each strain between the ID twins.

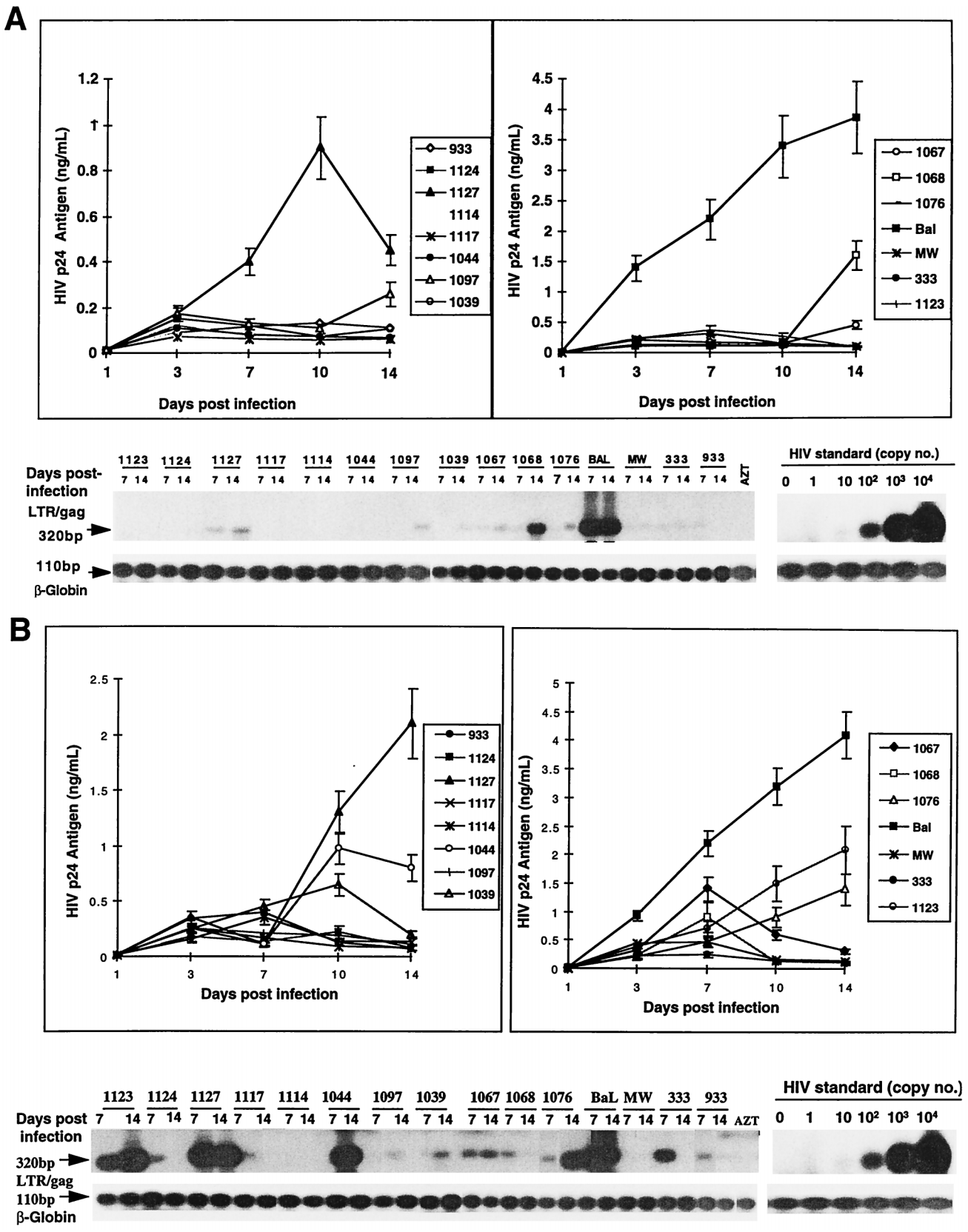


FIG. 2. Phase I: URD pairs. Shown are kinetics and levels of replication measured as HIV DNA and EC p24 antigen of 15 primary HIV isolates in 5-day-old MDM from URD pairs URD1 (A) and URD2 (B). In URD1, only strains BaL, 1127, and 1068 replicated to high levels; all others were low replicating. In URD2, note the marked discordance for strains 1044, 1039, 1068, 1067, 1076, and 1123. Replication kinetics and experimental conditions were as for Fig. 1.

external primer pair was NV3-1 (CAACTGCTGTTAAATGGCAGTCT, positions 6985 to 7008; using HIV-1 pNL43) plus NV3-2 (ACTGTGCATTACAAT TTCTGGGTC, positions 7316 to 7339). The V3 internal primer pair was NV3-A (GCAGTCTAGCAGAAGAAG, positions 7002 to 7019) plus NV3-B (TGGG TCCCCTCCTGAGGA, positions 7304 to 7321). The *vpr* external primer pair was NVPR-1 (GAAGATAAAGCCACCTTTGCC, positions 5511 to 5533) plus NVPR-2 (GCAGTCTTAGGCTGACTTCCT, positions 5871 to 5891). The *vpr* internal primer pair was NVPR-A (GCCACCTTTGCCTAGTGTTAAG, positions 5520 to 5541) plus NVPR-B (TTAGGCTGACTTCCTGGATGC, positions 5865 to 5886). Both rounds of PCR were preceded by a denaturation step at 94°C for 5 min and ended by extension at 72°C for 7 min. Samples (10 μl) of the second round of PCR were electrophoresed on 1.5% agarose gel, detecting DNA fragments of 320, 367, 285, 606, and 654 bp for the V3 region, *vpr*, *vif*, *vpu* and *nef*, respectively. PCR products were precipitated with a solution containing 26.7% polyethylene glycol 8000, 0.6 M sodium acetate (pH 5.2), and 6.5 M $MgCl_2$, washed twice with 95% ethanol, air dried, and reconstituted with an appropriate amount of sterile water. The DNA concentration was spectrophotometrically quantitated; and 100 to 300 ng was used for sequencing with 10 pmol of sense primers and confirmed with the antisense primers. The purified PCR products were sequenced by the dye-deoxy terminator technique in an ABI model 373A automated DNA sequencer. Sequence alignment was done with Clustal W, and phylogenetic trees were generated by Phylip (ANGIS facility, University of Sydney).

Statistical analysis. The kappa values, which measure agreement between two sets of data adjusted for chance association, were determined by previously described methods (26). The same sets of data were analyzed by Spearman rank correlation, and the coefficient was determined by using SPSS for Windows (release 7.0). For two-by-two comparisons, *P* values were determined by Fisher's exact test.

Nucleotide sequence accession numbers. The output sequences from two twin pairs and two URD pairs infected with five HIV isolates after infection of macrophages from two twin pairs and two URD pairs have been assigned the following GenBank accession numbers: AF133342 to AF133381 for the V3 region and AF133382 to AF133421 for the *vpr* gene, respectively.

RESULTS

Genotyping of twins and URDs to examine identity. According to maternal interview, we provisionally designated eight twin pairs as ID and three as NID (in physical features).

(i) HLA-DR typing. As shown in Table 1, seven of the eight twin pairs were shown to be identical but HLA-DR typing of the twin pair BH/DH was discordant: (DH, 08, 0101/2/4; BH, 04, 0101/2/4). One (MG/RG) of the three provisionally NID twin pairs was identical by HLA typing. The other two NID twin pairs (GS/DS and PG/NG) were confirmed to be so with both assays.

(ii) Zygosity testing. Further testing of the 11 twin pairs for microsatellite markers showed insignificant differences in most markers for location of their alleles in the same seven of the eight provisionally ID twins and one-third of the provisionally NID twins (Table 1). Therefore, one pair in each group was actually misclassified by maternal interview and were really NID and ID, respectively, by both HLA-DR and zygosity typing. Therefore, overall three twin pairs (DH/BH, GS/DS, and NC/PC) showed significant differences with the microsatellite markers D1S235, D1S356, D4S192, D6S89, TGFA, and TYRP2A, confirming that they were NID and refining the results of HLA-DR typing.

Coreceptor utilization by HIV isolates. All four primary isolates and HIV-BaL used in phase II of the study were tested for HIV infection of a panel of HOS.CD4 cells transfected with CCR1, CCR2B, CCR3, CCR4, CCR5, CXCR4, Bonzo/STRL33, and Bob/GPR15 cDNAs. Infection was determined by DNA PCR and concentration of supernatant p24 antigen in cultures. All strains except one utilized solely CCR5 as the coreceptor; the exception, strain 1192, used CCR3 in addition to CCR5 (data not shown).

Replication of primary isolates of HIV-1 in monocytes and MDM from twins and unrelated donors. The experiments were conducted in two phases. The first phase was a survey of sufficient primary HIV-1 strains to establish the different patterns of replication in both monocytes and MDM from differ-

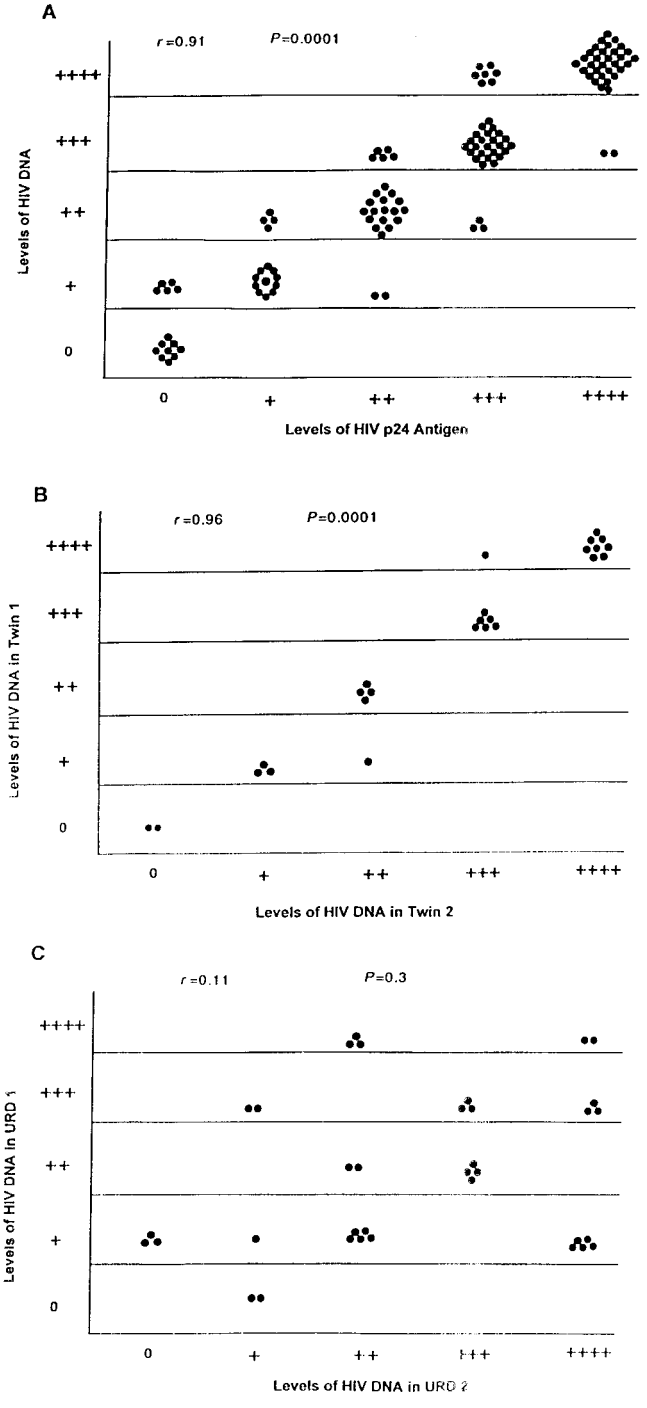
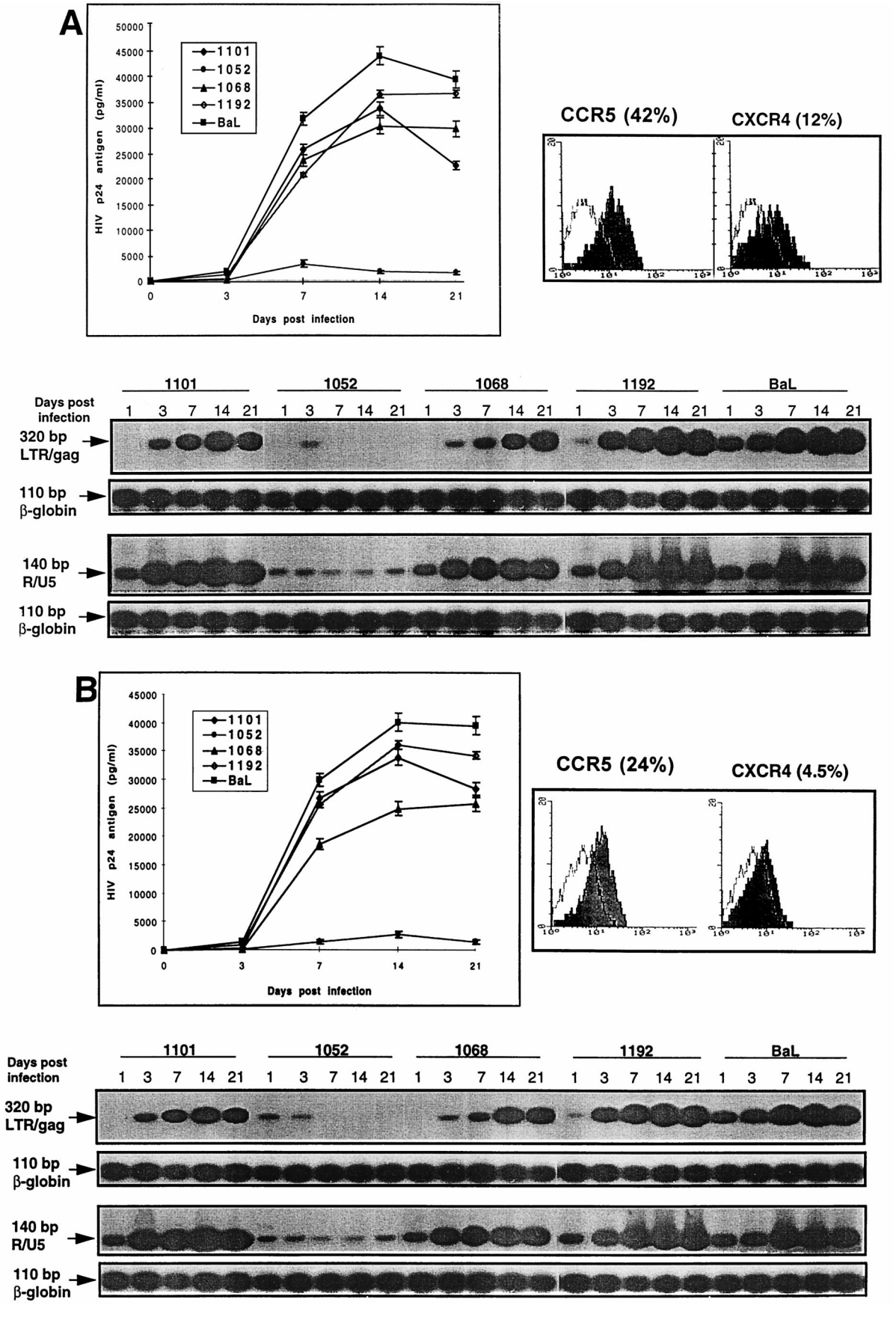


FIG. 3. Phase II: scattergrams demonstrating the correlation of levels of replication of five HIV-1 strains in macrophages from five pairs of ID twins and seven pairs of URDs as measured by HIV p24 antigen and intracellular HIV DNA. Levels of p24 antigen at their peak at either day 7 or day 14 after infection and peak HIV DNA levels at day 7 were classified as described in Materials and Methods. (A) Correlation between HIV p24 antigen and HIV DNA from all host cell-HIV pairs (a total of 120 host MDM-HIV combinations); high concordance ($r = 0.91$, $P = 0.0001$). (B) Correlation between levels of HIV replication within identical twin pairs (twin 1 versus twin 2) measured as intracellular HIV DNA (a total of 25 host MDM-HIV combinations); high concordance ($r = 0.96$, $P = 0.0001$). (C) Correlation between levels of HIV replication within unrelated donor pairs (URD1 versus URD2) measured as intracellular DNA (a total of 35 host MDM-HIV combinations); low concordance ($r = 0.11$, $P = 0.3$).


A
HIV p24 antigen (pg/ml)
Days post infection
1101
1052
1068
1192
BaL
CCR5 (42%)
CXCR4 (12%)
Days post infection
320 bp LTR/gag
110 bp β-globin
140 bp R/U5
110 bp β-globin
B
HIV p24 antigen (pg/ml)
Days post infection
CCR5 (24%)
CXCR4 (4.5%)
Days post infection
320 bp LTR/gag
110 bp β-globin
140 bp R/U5
110 bp β-globin

ent donors and in twins. The second phase concentrated on just five HIV strains with higher MOIs, using earlier time points for comparison of the initiation and complete RT products (cDNAs) to determine the site of action of host genetic effects during first round of replication. CCR5 genotype, CCR5 and CXCR4 expression, and HIV replication were also correlated for each donor and donor pair.

(i) Phase I. Monocytes or MDM from three pairs of ID twins and three pairs of URDs were infected with a large panel of 14 primary isolates and with the laboratory adapted strain HIV-BaL. Two of the isolates (1127 and BaL) always replicated to high levels, and seven (933, 1114, 1117, 1124, MW, 1097, and 333) produced either undetectable or very low levels of p24 antigen at all time points in all donor monocytes/MDM. However, six primary isolates (1123, 1039, 1044, 1067, 1068, and 1076) varied in the level of productive infection from low to intermediate to high, depending on the host cell donor. Other primary isolates used in the second phase of the study were also classified in a similar fashion as high (1192), variable (1101), or low (1052) (54).

In the three pairs of ID twins, the hierarchies of replication of HIV strains in both monocytes and macrophages were identical and the kinetic curves of p24 were very similar. BaL, 1127, and 1123 replicated to high levels in both members (Fig. 1A and B) of the twin pair, with the others all being low replicating. In contrast, the patterns of replication of the 15 isolates in three pairs of unrelated donors were discordant, as shown with the pair URD1 and URD2 (Fig. 2). For example, five HIV strains replicated to high or intermediate levels in URD2 but to lower levels in URD1. In contrast, strain 1068 replicated to higher levels in URD1. Only three produced similar levels of p24 in both URD1 and URD2. Two of these (BaL and 1127) were consistently high replicators in all donor cells.

HIV DNA was quantified as complete RT products by hot PCR to detect HIV DNA replication at 7 and 14 days, as earlier studies showed HIV DNA levels to peak and plateau at 7 days (54). Overall there was a high correlation between HIV DNA level and EC HIV p24 antigen ($r = 0.88$, kappa = 0.57, $P = 0.001$ [data not shown]), suggesting that any restriction to HIV replication occurred at or before RT. However, in some HIV-monocyte/MDM cultures where production of HIV antigen was very low or undetectable, HIV DNA levels were moderate, indicating that restriction of replication could also occur after RT. This was observed in 30% of low productive infections of monocytes but was less common in 5-day-old MDM (5% [see below]).

Within the three ID twin pairs, both the HIV DNA and p24 antigen levels were similar for each primary isolate and BaL ($r = 0.97$, kappa = 0.88, $P = 0.001$ [data not shown]). The maximum differences in the productive infection were <1 ng of p24 antigen per ml and <500 copies of HIV DNA by PCR. However, within the three pairs of URDs, the HIV DNA and p24 antigen levels for the variable strains usually differed significantly ($r = 0.01$, kappa = −0.09, $P = 0.2$ [data not shown]). These concordant patterns of HIV kinetics between ID twins and discordant patterns in URDs were observed at any stage of maturation of monocytes into MDM (1, 3, and 5 days [see below]), although the differences were greater in 1-day-old monocytes (where HIV production is generally lower) (54, 75).

Overall, phase I showed that one-third of the 15 isolates showed variable replication kinetics for HIV DNA and p24 antigen in different donors, but this variability was almost eliminated in ID twins. Although HIV DNA was initially measured at a fairly late stage (7 and 14 days) in phase I, the high correlation with p24 antigen suggested that the host genetic effect acted before or during RT (or possibly on intercellular spread). These hypotheses were tested more intensively in phase II.

(ii) Phase II. MDM at an intermediate stage of differentiation (day 3) were infected with four primary isolates selected for high (1192), variable (1068 and 1101), or low (1052) levels of replication and also with the laboratory-adapted HIV strain BaL. All were infected with the same MOI of 0.1 $TCID_{50}$/cell, which was higher than in the first phase of experiments, to facilitate detection of HIV DNA during the first cycle of infection before virus spread. The kinetics of HIV p24 antigen and HIV DNA were compared between the members of the five ID twin pairs, three NID twin pairs (data not shown), and seven pairs of URDs. However, HIV DNA was measured earlier in this phase, at days 1, 3, and 7 after infection. As expected, 1192 replicated to high levels and 1052 replicated to low levels in most (but not all) donors, whereas 1068 and 1101 showed variable levels. The patterns of HIV replication were concordant within all of the ID twin pairs, with the same hierarchy of peak p24 antigen levels and similar levels of HIV DNA obtained at various time points, especially for the variable HIV strains. The overall correlation within the twin pairs for each of the five isolates was high ($r = 0.98$, kappa = 0.90, $P = 0.0001$ [Fig. 3B]), especially for the variable strains as shown in Fig. 4. The levels of incomplete and complete HIV RT products (HIV cDNA) and p24 antigen levels were much less variable for 1068 and 1101 within twins than within the URD pair (Fig. 4 and 5, respectively). In five of seven URD pairs, there was marked discordance in HIV DNA levels. Overall, there was a very poor correlation within all URD pairs ($r = 0.29$, kappa = 0.04, $P = 0.6$ [Fig. 3C]).

In some of these pairs (three ID twin pairs and two URD pairs) hot PCR was repeated with primers detecting the initiation products of HIV RT, showing similar correlations in most cases (Fig. 4 and 5 show data for strain 1068), suggesting a restriction to productive infection at a stage pre-RT. However, with 1052, a consistently low replicating isolate as measured by p24 antigen, full-length HIV DNA levels were low but the incomplete DNA levels were higher, suggesting a restriction both before and during RT (Fig. 5).

Overall, there was a very high correlation between HIV DNA levels (at days 3 and 7) and EC HIV p24 antigen levels (at days 7 and 14) in each of the HIV-MDM cultures ($r = 0.93$, kappa = 0.70, $P = 0.0001$ [Fig. 3A]), similar to the results in phase I. There was a high correlation of >90% between HIV DNA levels and p24 antigen levels at day 7 and 14 for each strain. However, in HIV-MDM cultures where productive in-

FIG. 4. Phase II: ID twin pairs. Shown are correlations of the kinetics and levels of replication of five HIV isolates with the proportions of CCR5-expressing cells in 3-day-old MDM from ID twin pair CG (A) and PG (B). Three-day-old MDM were infected with four primary isolates and BaL at a higher MOI of 0.1 $TCID_{50}$/cell, and replication kinetics were examined by assays for EC p24 antigen and DNA PCR. HIV DNA was detected by quantitative PCR using two sets of primer pairs, one detecting 320-bp DNA fragments spanning the LTR-*gag* region of nearly full-length HIV DNA and the other detecting 140-bp fragments of the initiation products, in the R-U5 region of the LTR, of RT to assess entry at day 1 and thereafter. Membrane CCR5 and CXCR4 expression by MDM was examined by flow cytometry using specific monoclonal antibodies 2D7 (LeukoSite) for CCR5 and 12G5 (R & D Systems) for CXCR4. The proportion of cells expressing CCR5 and CXCR4 above control levels is shown. Note the similarity in kinetics between peak of HIV p24 antigen and HIV DNA with variable isolates 1068 and 1101. HIV DNA levels for strain 1052 were low for the first 3 days and then decreased concordantly in both members of the twin pair. EC p24 antigen was undetectable at all stages.

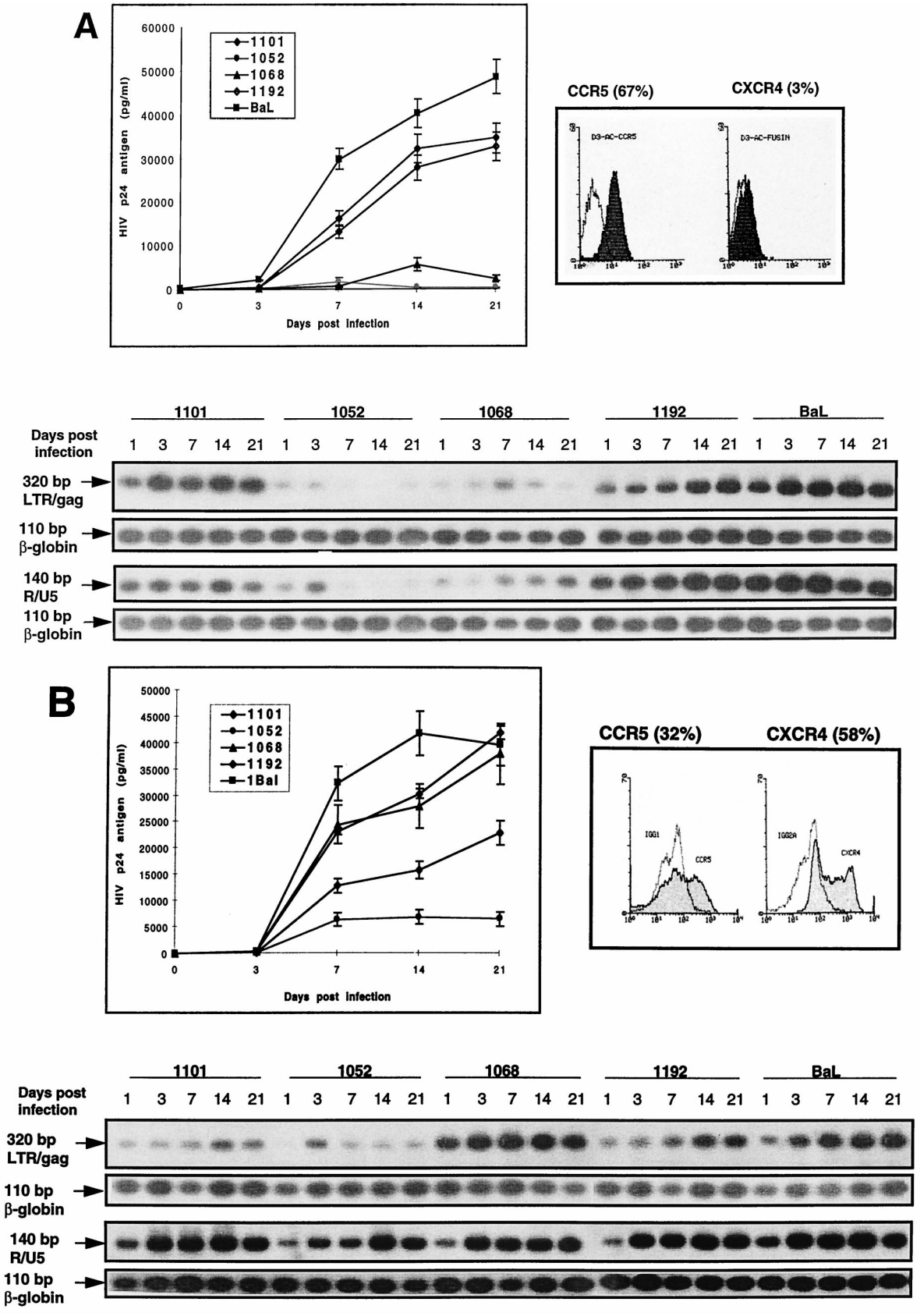


FIG. 5. Phase II: URD pairs. Shown are correlations of the kinetics and levels of replication of five primary HIV isolates with the proportions of CCR5 and CXCR4-expressing cells in 3-day-old MDM from URD pairs. Experimental conditions are as for Fig. 4. Note that even with a high proportion of cells expressing CCR5, there is pre-RT restriction of replication of the CCR5-using strain 1068 (panel A).

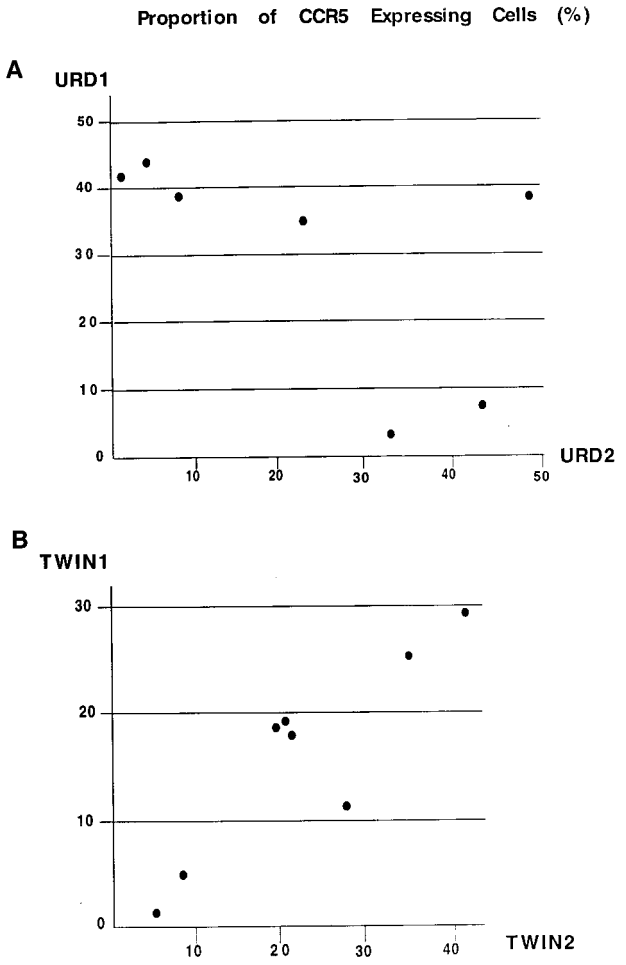


FIG. 6. Correlation of proportions of 3-day-old MDM expressing CCR5 within URD pairs (A) and within ID twins (B). Membrane CCR5 and CXCR4 expression by MDM was examined as described in the legend to Fig. 4.

fection was persistently very low or undetectable, 5% (15 of 300) showed moderate levels of HIV DNA (even at day 3). Thus, the restriction to replication in this 5% appeared to be between the stage of near completion of RT and production of extracellular virus (i.e., post-RT).

In some HIV-MDM cultures, extracellular fluid levels of reverse transcriptase of infectious HIV, HIV DNA, and p24 antigen levels were all tested, and a good correlation was observed (data not shown).

Genotyping and expression of CCR5 in macrophages from twins and URDs. The presence of the CCR5 Δ32 deletion in twin pairs and URDs was sought by PCR to determine the heterozygous or homozygous genotype. No homozygotes for the Δ32 mutation were found, but two ID twin pairs (KW/MW and RG/PG) and three URDs (CR, NS, and SH) were identified as heterozygotes (Table 1). The heterozygous state did not appear to alter the level of replication of the two high-replicating isolates in both ID twins and URDs.

The proportions of cells expressing CCR5 were measured by flow cytometry and found to be more similar within ID twin pairs (Fig. 6B) than within URD pairs (Fig. 6A) ($r = 0.81$ and $P = 0.01$, for identical twins; $r = 0.58$ and $P = 0.2$ for URDs). There was wide variability in the proportion of CCR5-expressing cells in both the wild type and the CCR5 Δ32 heterozygotes, ranging from <1 to 67% for the wild-type CCR5 and from 18 to 32% for CCR5 Δ32 heterozygotes. Hence, the differences between the means were not significant ($P = 0.35$; Student's t test).

Importantly, there was also little correlation between the proportion of CCR5-expressing cells and the level of replication of HIV-1 isolates, as shown in Fig. 7. Moderate levels of productive infection were observed even at low levels of CCR5 expression, except for one URD where <1% of MDM expressed CCR5. No primary isolate replicated in these cells, and BaL replicated poorly, with low HIV DNA and p24 antigen levels. The very low HIV DNA level was consistent with a block at entry (Fig. 7A).

Sequences of the output strains from MDM infected with primary HIV-1 isolates. The 15 viral strains initially used to infect three pairs of ID twins and by URDs were sequenced for the V3 region of the envelope gene and *nef*, *vpr*, *vpu*, and *vif* genes. Sequence alignment and slanted cladograms were constructed and inspected for clustering of consistently low, consistently high, or variably replicating strains. As the restriction to replication at virus entry was most common, the V3 regions were the most carefully examined. However, as post-RT restriction was also observed, the accessory gene sequences for these isolates were examined too.

There was no consistent clustering of the five low- or three high-replicating strains for any of the five genes, indicating there was no consistent sequence motif in any individual gene facilitating high or low replication (data not shown). However, the low-replicating strain 1052 clustered separately from the high or variable strains in the V3 region (Fig. 8A).

The sequences of the predominant output strains from HIV-infected macrophages from the first two pairs of ID twins and the first two pairs of URDs (in phase II) were also compared as cladograms for V3, *vpr*, and *vif*. There was a closer association of the V3 (Fig. 8A) and *vpr* (Fig. 8B) sequences of strains from ID twins than from URDs. For both V3 and *vpr*, the strains from the ID twin pairs clustered on the same branch on 7 of 10 occasions but did so for the two URD pairs (1 versus 2 and 3 versus 4) on 0 of 10 occasions ($P = 0.0015$, Fisher's exact test). This discrepancy could not be explained by the differences in viral replication affecting sequence variability, as sequence variability within twin pairs was reduced in both high- and low-replicating strains. These experiments strongly suggest that host factors influence viral strain selection from quasispecies.

DISCUSSION

In phase I of this study, we confirmed and extended the findings of our previous study with a much larger panel of 15 primary HIV isolates used in both 1-day-old monocytes and 5-day-old MDM from three ID twin pairs and three URD pairs. The primary isolates could be classified as consistently high, consistently low, or variably replicating isolates in monocytes and macrophages. These patterns were confirmed in phase II, demonstrating that isolates selected as high, low, or variable retained these characteristics in a further set of experiments. The variable strains identified in phase I were obviously the strains most affected by host cell genetics, and this continued to be true for variable strains in phase II. A host cell genetic effect was also observed at various stages of maturation of monocytes into macrophages, despite the increasing permissivity for tropism and productive infection in more advanced stages of maturation (days 3 to 7 after adherence). Using quantitative highly sensitive hot PCR to detect and quantify the first round of HIV RT products during replication (within the first 3 days) we observed in both phases of the study a high

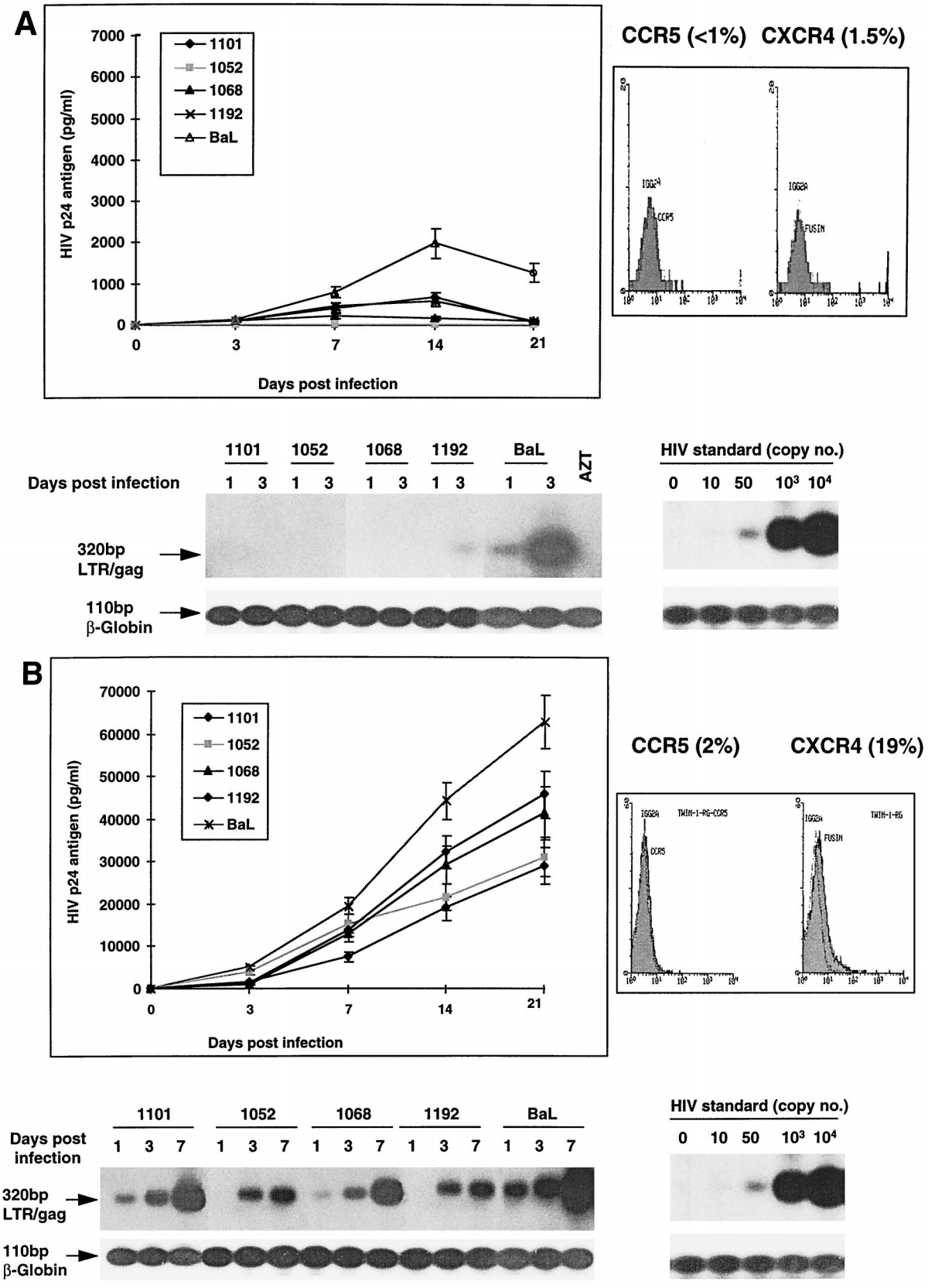


FIG. 7. Phase II: URDs. Shown are correlations of levels of HIV replication measured as HIV DNA and EC p24 antigen of 5 HIV-1 strains with CCR5 and CXCR4 expression in 3-day-old MDM from URD pairs (A and B). There were marked restriction of HIV BaL and complete inhibition of the primary isolates (A) when the level of expression of CCR5 was below the threshold of detection, but in panel B, where only 2% of cells expressed CCR5 above control levels, all strains entered the MDM and replicated well. Experimental conditions were as for Fig. 4.

and significant correlation between HIV DNA levels and EC p24 antigens in isolates which consistently replicated to intermediate or high levels and in most of the low-replicating isolates. Therefore, the host cell restriction to HIV productive infection in variable isolates occurred prior to RT. This was verified by showing a similar correlation between early and late RT products and EC p24 antigen levels. The same pattern was seen in low-replicating viral strains, regardless of host genetics, except isolate 1052. This consistently low replicating isolate showed higher levels of HIV DNA produced by initiation RT primers than levels produced by complete RT primers within the first 3 days (first cycle), suggesting restriction during RT, probably determined by the viral genotype. High-replicating isolates demonstrated HIV DNA levels exceeding 10,000 copies/million cells, whereas in low-replicating isolates HIV DNA ranged between 100 to 500 copies/million cells. In both phases of the study, HIV DNA levels showed a high and significant correlation within members of the ID twin pairs and discordance between URD pairs in most cases, especially with the variable strains. In 5% of infections with low-replicating viruses in MDM and 30% of such infections in monocytes, there was a substantial nonproductive infection, with undetectable levels of HIV p24 antigen but moderate levels of HIV DNA. In most cases, these HIV DNA levels were significant for several days and then declined.

Taken together, these findings indicate that the major restriction to HIV replication in monocytes and macrophages occurs pre-RT, complementing previous work from our laboratory and others (51, 73). Furthermore, there is clearly a group of isolates which constitute approximately one-third to one-half of primary isolates (this study and reference 54) whose replication is markedly influenced by host cell genetics, and the major site of impact of this genetic effect is also pre-RT. We and others have previously shown (12, 36, 51, 56) that the site of restriction in monocytes/macrophages is likely to occur after binding to CD4. The results of the present study suggest that the major host genetic restriction effect is also after binding to CCR5. The likely site of restriction is later during viral entry or uncoating (7).

Membrane CCR5 levels on T lymphocytes have been reported to vary markedly between individual donors (49), and here we show this is also true for macrophages at the same state of differentiation (3 days after adherence). This variability appears to be genetically determined in macrophages, in view of the significant correlation between CCR5 expression within twin pairs but not within URD pairs. Surprisingly, there was a poor correlation between level of productive replication and CCR5 expression except at very low levels. HIV (especially HIV DNA) replication could be seen at very low or undetectable levels of CCR5 expression in MDM, suggesting that the threshold of CCR5 receptor expression which restricts HIV entry is lower than that detectable by flow cytometry. In view of this lack of correlation of threshold of CCR5 with HIV DNA levels, it seems likely that there may be unidentified host cell genes affecting the pre-RT stage of HIV replication, especially in view of the correlation between low, intermediate, and high levels of HIV DNA and levels of EC p24 antigen. The marked differences in these HIV DNA and p24 antigen levels in the variable isolates between URDs and between members of the non-ID twin pairs suggest that it may be possible to define these gene clusters further. The use of as high an inoculum as possible and highly sensitive PCR within the first 3 days together with the excellent correlation between day 3 HIV DNA levels (during the first round of HIV replication in macrophages) and p24 antigen levels at days 7 and 14 argue for a predominant effect on HIV entry other than spread through the cell cultures. Nevertheless, host cell genetic effects on HIV spread are to be examined by in situ PCR as an extension of these experiments.

As discussed above, CCR5 appears to be the major coreceptor facilitating HIV entry into macrophages. However, isolates utilizing CXCR4, CCR3, CCR2b, Bonzo, and Bob have also been identified. The importance of CCR5 as a coreceptor for T lymphocytes has been demonstrated by the inability to infect primary T cells from almost all of the individuals who are homozygous for the Δ32 mutation. This has recently also been reported for HIV infection of macrophages (54, 62). However, some strains are able to circumvent the absence of functional CCR5 by utilizing other coreceptors such as CXCR4, CCR3, CCR2b, and STRL33 (Bonzo), and probably also GPR15 (Bob) and GPR1, or HIV strains infecting CCR5 Δ32 homozygous individuals (46).

There has been some debate about the importance of the CCR5 Δ32 heterozygous state in determining susceptibility to infection. Recent studies have demonstrated reduced amounts of CCR5 expression on the surface of mononuclear cells in heterozygotes and lower HIV load in vitro (21, 32, 45). Several studies have now also demonstrated a correlation between the heterozygous state and slower progression of HIV infection, suggesting at least some impairment of HIV replication in vivo, although it is not the sole determinant for long-term nonprogression of disease (11). In this study we sought evidence for an effect of the Δ32 mutation in the heterozygous state as an explanation for the marked host genetic effects on restriction of HIV entry and uncoating. In heterozygotes, there was a slight decrease in the mean proportion of MDM expressing membrane CCR5 antigen, but this was masked by high variability of expression. However, in the small number of heterozygotes in this study, there was no clear effect on the concentrations of EC p24 antigen or intracellular HIV DNA after infection of these cells with primary HIV isolates. In general, all primary isolates replicated to similar levels in heterozygotes and wild-type CCR5 cells.

Recently, evidence that the differences between high- and low-replicating isolates may also be due to restriction at stages following RT (33, 50, 69) or due to the variable rate in virus spread in cultures (71, 75) has been reported. In a minority of low or variably replicating viruses in this study, the restriction to replication was found to be post-RT, suggesting that some isolates are indeed controlled by post-RT host cell mechanisms (and possibly at the level of viral spread).

The current results resolve some of the controversy between differing reports of tropism for monocytes/macrophages occurring in the early stages of HIV replication or conversely in the late stages after RT. Restriction of replication clearly can occur at both stages, but the predominant effect with most clinical strains is pre-RT. Other studies have mostly used laboratory-adapted strains of HIV. Integration of proviral DNA has also been reported to be essential for viral transcription and productive infection in MDM (20). Therefore, nuclear importation into macrophages (which do not undergo mitosis), integration, or transcription may be defective or influenced by host cell genetics in some HIV strains. Furthermore, viruses with high replication efficiency could also be more responsive to activation of virus transcription induced by cytokines (such as tumor necrosis factor alpha, interleukin-1, and interleukin-6) or β-chemokines (37, 53, 58, 60, 77).

All 15 HIV strains used in phase I were sequenced for the V3 region, in view of its importance in HIV coreceptor binding and entry. They were also sequenced for the accessory genes *nef*, *vpu*, *vif*, and *vpr*, reported to influence HIV replication in macrophages, particularly at post-RT stages (6, 43). However,

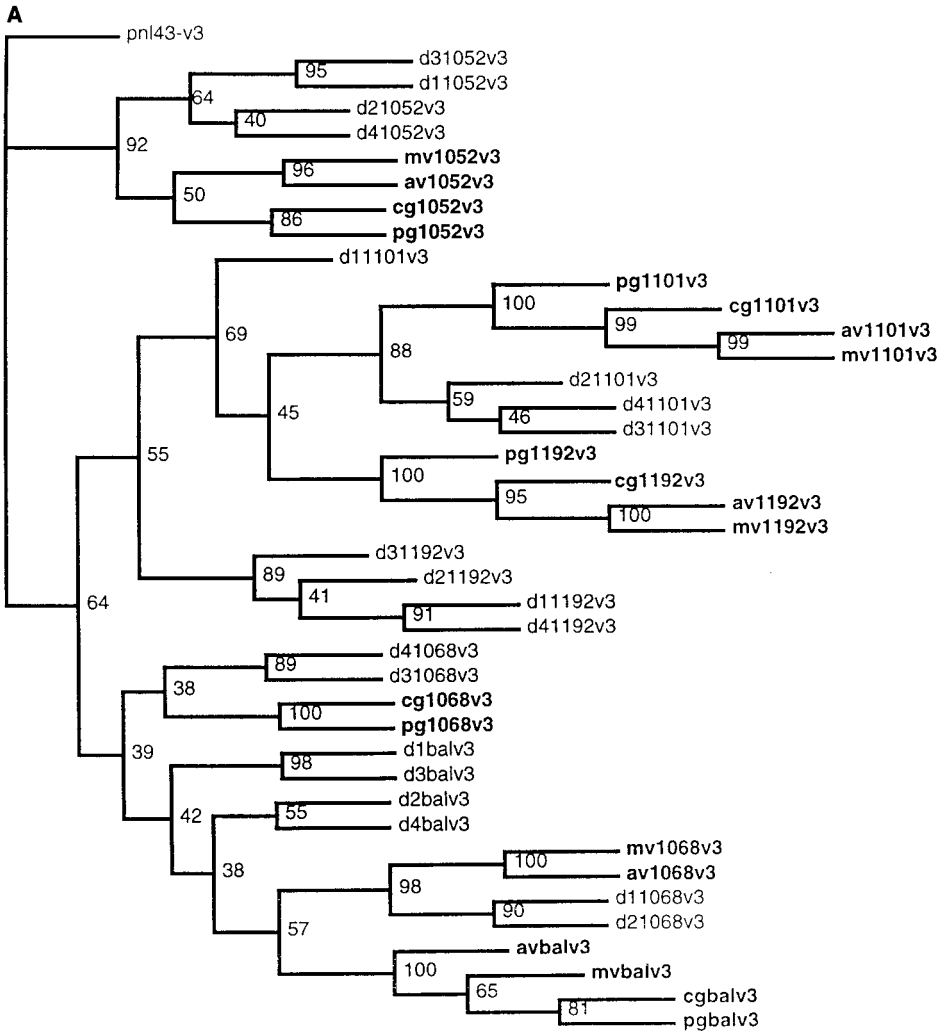


FIG. 8. Neighbor-joining distance tree of V3 region (A) and *vpr* gene (B) sequences compared within two twin pairs (AV/MV and CG/PG) and within two URD pairs (d1/d2 and d3/d4). Note that the sequences from each isolate tend to cluster together, independent of donor cell source, but that there was a closer association of V3 and *vpr* sequences from ID twin pairs than from URD pairs.

no common or similar V3 sequence motifs responsible for restricting HIV entry into macrophages were identified, nor did we find any common or similar accessory genes sequence motifs which may have accounted for post-RT-transcription restriction of replication.

After infection of macrophages from the first two pairs of ID twins and the first two pairs of URDs, the predominant output HIV strains were compared for the effects of host cell genetics on the selection of strains. This was apparent by inspection of the cladograms and showed a significant degree of association of the sequences from two regions of HIV strains within pairs of ID twins compared with that within the pairs of URDs.

These studies provide strong support for the notion of viral and host protein interactions at multiple levels within the viral replication cycle within the cells. Each of these interactions could provide a potential bottleneck for viral replication (e.g., very low levels of membrane CCR5 with or without the homozygous CCR5 Δ32 mutation). Hence, these bottlenecks may be defined by the interaction of a specific viral protein and a critical host protein. This is likely to define the selection of the predominant strain from within the infecting quasispecies. Therefore, it is not surprising that viruses emerging from the cells of identical twins are very similar, whereas virus output sequences from unrelated donors differ. As the bottleneck may occur at any stage, such a difference at any one gene may select different viral sequences appropriate to the host cell protein sequence. For example, where two donor cells have similar levels of expression of the same coreceptors on the cell membrane and the bottleneck is at the level of cell proteins involved in transport of the preintegration complex to the nucleus, this may select certain strains in the quasispecies through *vpr* rather than V3. Nevertheless, *vpr* and V3 sequences must be linked within the same genotype, unless recombination occurs, and this will usually be apparent in comparisons in unrelated donors as shown here. The importance of this type of selection in vivo compared with immune selection needs to be better defined.

This study demonstrated clearly that the predominant effect of host cell genetics on HIV replication in monocytes/macrophages was manifest as restriction of replication predominantly prior to RT. To a much lesser extent, there was also post-RT restriction. Although CCR5 appears to be required for HIV entry into macrophages, our findings suggest that this is likely to be only one of several host factors determining the level of

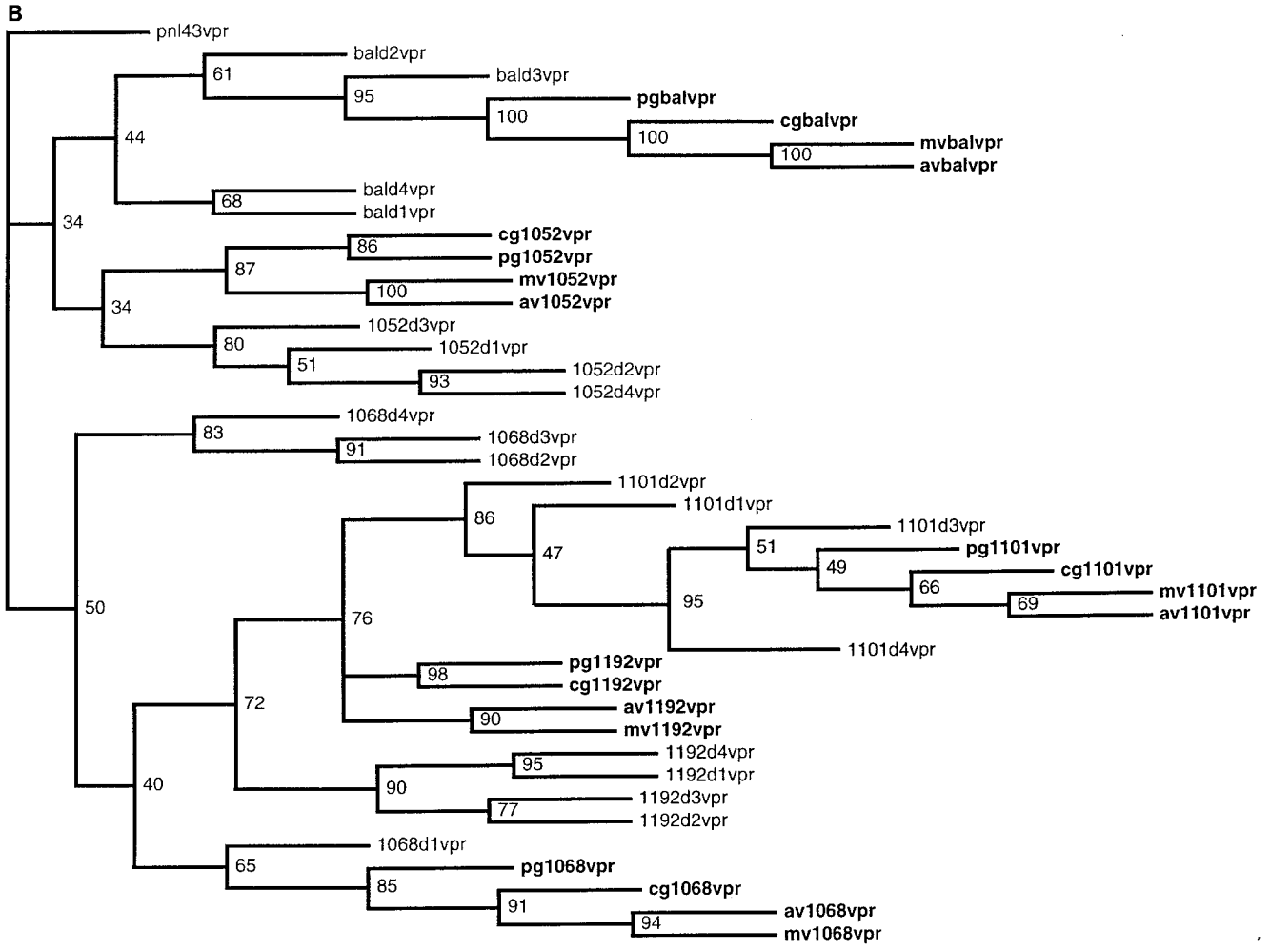


FIG. 8—*Continued.*

HIV replication in these cells. We are currently expanding this approach to involve T lymphocytes and more ID/NID twin pairs and family studies to define other host cell gene clusters influencing the early stages of HIV replication. Preliminary results with NID twin pairs have shown a host genetic effect intermediate between ID twins and URD pairs, as expected.

ACKNOWLEDGMENTS

We thank Nick Martin's group from QIMR for assistance in the zygosity assay and LeukoSite, Inc., for providing the monoclonal antibodies to CCR5. The twins were recruited through the Australian NHMRC Twin Registry with advice and assistance from John Hopper. We also extend our thanks to D. Littman for providing HOS cells and Karen Byth for assistance in statistical analysis.

This work was supported by ANCARD through the Australian National Centre for HIV Virology Research.

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