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Spontaneous Mutations in the Human Immunodeficiency Virus Type 1 *gag* Gene That Affect Viral Replication in the Presence of Cyclosporins

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Human immunodeficiency virus type 1 mutants that are resistant to inhibition by cyclosporins arise spontaneously in vitro during propagation in a HeLa-CD4$^+$ cell line in the presence of a nonimmunosuppressive analog of cyclosporin A. Interestingly, the phenotype of all of the mutants examined is drug resistant and drug dependent, with both cyclosporin A and its analog. Four independently isolated mutants have been analyzed genetically by construction of recombinant proviruses in the NL4-3 parental strain background and subsequent testing of the chimeric viruses in HeLa cells. The cyclosporin-resistant, cyclosporin-dependent phenotype consistently transfers with a 1.3-kb fragment of *gag*, within which the four mutants share one of two possible single amino acid exchanges in a proline-rich stretch in the capsid domain of Pr55^{gag}. These mutants provide the first evidence that mutations in human immunodeficiency virus type 1 *gag* confer resistance to cyclosporins; however, replication is conditional on the presence of the drug. In the T-cell line CEM, replication of the recombinant mutant viruses is also cyclosporin dependent. The drug-dependent replication in HeLa cells is stringent, and in the absence of cyclosporin only revertant viruses with the parental phenotype grow out of cultures infected with cyclosporin-dependent virus. In at least one isolate examined, the revertant phenotype appears to be due to suppressor mutations near the proline-rich region.

For several years it has been known that the fungal metabolite cyclosporin A (CsA), which is more familiar as a widely used immunosuppressive drug, inhibits human immunodeficiency virus type 1 (HIV-1) replication (11, 23). More recently, it has been shown that other cyclosporins (synthetic or natural analogs of CsA) that lack immunosuppressive activity nonetheless retain the ability to inhibit virus replication (2, 4, 16), and it was observed that the antiviral activities of a number of different cyclosporins correlate with the ability to bind cyclophilin A (CyPA), the major cytosolic binding protein for cyclosporins (10). In particular, one of the nonimmunosuppressive cyclosporins, the Melle-4 analog (termed SDZ NIM 811), has been examined in this context (16, 22). Interestingly, a completely independent line of investigation, namely, the seeking of cellular protein binding partners for HIV-1 Gag proteins as potential cofactors for virion assembly and events early postinfection, has also identified and focused interest on the CyPs (13). Subsequently, several observations have led to the idea that the interaction between Gag and CyP A is necessary for HIV-1 replication and that the mechanism of cyclosporin inhibition is through disruption of this interaction: (i) cyclosporins inhibit Gag-CyP association in vitro (4, 13); (ii) cyclosporins diminish CyP incorporation in virus particles, and this incorporation is mediated by *gag* (8, 20); and (iii) a mutation in *gag* that diminishes Pr55^{gag}-CyP binding in vitro and incorporation of CyP in virus particles also affects infectivity (8).

In order to better understand the effects of cyclosporins on the replication of HIV-1, we have addressed the problem of possible resistance development during virus replication in the continual presence of cyclosporins over relatively long periods of time. This issue is important in light of the current interest in these compounds as potential antiviral therapeutic agents. Furthermore, the selection of spontaneous non-replication-defective mutants under conditions that, according to experimental evidence, do not favor Gag-CyP interactions may provide useful tools for probing this question. We describe here the isolation of cyclosporin-resistant and -dependent (Cs$^{R/D}$) mutants of HIV-1 strain NL4-3 that spontaneously arise during replication in HeLa-CD4$^+$ cells, their phenotype in the CEM T-cell line, and the genetic characterization of the mutants. A revertant isolate that reacquired the cyclosporin-sensitive phenotype is also described.

MATERIALS AND METHODS

Cyclosporins. CsA and the nonimmunosuppressive, 4-substituted analog SDZ NIM 811 ([Melle-4]cyclosporin) (22) are natural metabolites of the fungus *Tolypocladium niveum*.

Cells and viruses. HeLa-CD4$^+$-LTR-β-gal cells (12) were grown in Dulbecco's modified Eagle minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 0.2 mg of geneticin per ml, and 0.1 mg of hygromycin B per ml. These cells were obtained from M. Emerman through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases). COS-1 cells were cultured in Dulbecco's modified Eagle minimal essential medium supplemented with 5% heat-inactivated fetal calf serum and 2 mM L-glutamine. CEM cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 μg of geneticin per ml. COS-1 cells and CEM cells were obtained from the American Type Culture Collection.

The HIV-1 proviral recombinant clone pNL4-3 (1) was obtained from the AIDS Research and Reference Reagent Program. Viruses were rescued from pNL4-3 and other proviral recombinant plasmid DNA clones by DEAE-dextran-mediated transfection of COS-1 cells by a modification of a previously published method (5). Cultures were inoculated the day before transfection in 35-mm-diameter wells in multiwell plates at 3×10^5 cells per well. Transfection of DNA was performed by incubating cells at 37°C for 90 min in 1 ml of complete growth medium containing DNA (250 ng/ml) and DEAE-dextran (60 μg/ml); then 10 μl of 10 mM chloroquine was added and incubation was continued for an additional 30 min. The cells were subsequently shocked with 12.5% dimethyl sulfoxide in 0.9 ml of prewarmed, complete growth medium for 2.5 min at room temperature. At 24 h after transfection, the growth medium was replaced, and virus was collected

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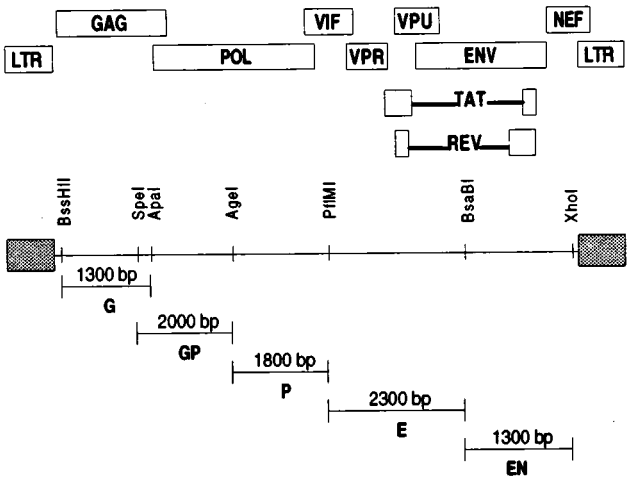

FIG. 1. Construction of recombinant MC–NL4-3 proviruses by substitution of mutant MC segments for the homologous sequences in pNL4-3 vectors. At the top is depicted a genetic map of HIV-1, below which is shown the relative positions of the restriction enzyme sites used to prepare vectors and inserted DNA fragments (described in detail in Materials and Methods). Each of the five segments of DNA (G, GP, P, E, and EN) amplified from mutant proviral DNA is shown at the bottom, with the length of each segment indicated. LTR, long terminal repeat.

overnight. For standard virus stocks with higher titers, HeLa cells were infected with virus from COS-1 cells, and 4-h supernatants from highly infected HeLa cultures were collected and stored frozen in aliquots at −80°C; the $p24^{gag}$ concentration in these stocks was measured, and they were used in all dose-response and kinetic analyses of cyclosporin effects on virus replication. For the $Cs^{R/D}$ mutant viruses, high-titer stocks were collected in medium containing, and from cultures grown in the presence of, 1 μg of SDZ NIM 811 per ml.

$p24^{gag}$ ELISA and infectivity assays. Virus titers were assayed by enzyme-linked immunosorbent assay (ELISA) in clarified culture supernatants with a murine monoclonal anti-$p24^{gag}$ antibody (Polimun, Vienna, Austria) as a coating reagent, alkaline phosphatase-conjugated polyclonal rabbit anti-$p24^{gag}$ (generously provided by H. Jaksche, Sandoz Forschungsinstitut, Vienna, Austria), and purified recombinant $p24^{gag}$ (Intracel, London, United Kingdom) as a standard. Following a colorimetric phosphatase reaction (Bio-Rad, Richmond, Calif.), the A_{405} was measured (Labsystems Multiscan MCC/340). The $p24^{gag}$ concentration was correlated with the infectious titer by endpoint titration assays; in the case of NL4-3 collected from, and assayed in, HeLa cells, 1 μg of $p24^{gag}$ corresponded to approximately 10^5 infectious units. The infectious titer was also estimated visually by counting blue-stained cells and syncitia in cultures infected with serial virus dilutions after performing a cytochemical β-galactosidase assay described for the HeLa cell line used for infection (12).

Isolation of cyclosporin-resistant and -dependent mutant viruses. NL4-3 was grown in HeLa cells for 6 weeks in medium containing 3 μg of SDZ NIM 811 per ml by passage of culture supernatants each week onto fresh, uninfected cells. Cultures were set up in several 35-mm-diameter wells, and the supernatants from the individual wells were kept isolated from one another during the entire serial passage protocol to allow independent mutant derivation in the different wells. Growth medium from uninfected cultures was also carried through the passage protocol as a control for cross-well contamination. At the end of each passage, before transfer of the supernatant to fresh cells, the infected cultures were fixed and the number of infected cells was estimated by the cytochemical β-galactosidase method; this value was in the range of 1,000 to 10,000 infected cells per culture at the end of the passage. At the end of the 6-week passage, each supernatant containing virus was purified twice by endpoint dilution in HeLa cells in medium containing 3 μg of SDZ NIM 811 per ml.

Construction of recombinant mutant–NL4-3 proviruses and testing of the phenotype. Chimeric proviruses were constructed by substitution of DNA segments, which were amplified by PCR from the mutants, for the homologous segment in pNL4-3. A total of five DNA segments were selected, each one defined by two unique restriction enzyme sites (Fig. 1). Synthetic oligonucleotides flanking these sites were used as PCR primers to amplify mutant proviral DNA, which was extracted from the nuclei of HeLa cells infected with endpoint-diluted viruses. The amplified DNA segments were digested with the respective restriction enzymes and then ligated with pNL4-3 vectors prepared by restriction enzyme digestion and end dephosphorylation, by conventional cloning methods (17). Additional constructs made as controls were chimeric G fragments (Fig. 1) from five different amplification reactions of one of the mutant viruses and from NL4-3. The sequences of the oligonucleotides used as amplification primers were as follows: WP9, 5′-TCggCTTgCTgAAgCgCg-3′; WP11, 5′-TTCTgTCAATg gCCA-3′; WP12, 5′-gCAggAACTAgTACC-3′; WP13.2, 5′-TACACTCCATg TACCggTTC-3′; WP21, 5′-gAAgCAgAgCTAgAACTggC-3′; WP22, 5′-gTTg gTCTgCTAggTCAggg-3′; WP16, 5′-TCAgggAgTCTCCATAgA-3′; WP17, 5′-gCAgCCCAgTAATATTTg-3′; WP23, 5′-CgTTCAATggAACAggACCAT-3′; and SQP-3, 5′-CCATgCAggCTCACAgggTgTA-3′.

After construction of the recombinant provirus clones, virus was recovered in COS-1 cells as described above. The phenotypes of the recovered viruses were determined in HeLa cells by inoculation of two cultures, one of which contained 1 μg of SDZ NIM 811 per ml; virus recovered from pNL4-3 under the same conditions was included as a control. After 3 days, the cultures were given fresh growth medium, and 3 days later virus replication was assessed by $p24^{gag}$ ELISA. In selected recombinant proviruses showing the mutant phenotype, the DNA sequence of the entire substituted mutant segment was determined by the Sanger method with T7 DNA polymerase (U.S. Biochemicals, Cleveland, Ohio).

Tests for effects of cyclosporins on virus replication. Replication of virus in HeLa and CEM cells in the presence of various concentrations of CsA or SDZ NIM 811 was tested in simple 6-day dose-response assays. For tests in HeLa cells, on the day before infection 35-mm-diameter wells, each containing 1.5×10^5 cells in 2 ml of growth medium, were set up, and the next day cyclosporins and virus (1-ng $p24^{gag}$ equivalent from high-titer stocks [see above] per well) were added; after 3 days the growth medium was replaced with fresh medium (containing cyclosporin), and then 3 days later $p24^{gag}$ released in culture supernatants was measured. For tests in CEM cells, 35-mm-diameter wells containing 5×10^5 cells in 2 ml of medium were set up at the same time that cyclosporins and virus (1-ng $p24^{gag}$ equivalent per well) were added; after 3 days, the cultures were split 1:5, and 3 days later $p24^{gag}$ was measured.

A more detailed replication study was performed by kinetic analysis in the presence and absence of cyclosporins, with sampling of culture supernatants every 2 days over a 12- to 14-day period for $p24^{gag}$ released from infected cells. CEM cultures were set up in 35-mm-diameter wells, each containing 1.1×10^6 cells in 2 ml of medium, and split 1:2 every 2 days. The tests in HeLa cells entailed two sets of cultures: an initial (primary) set of infected cultures that were carried for 12 days and a second (secondary) set of cultures infected with the 6-day supernatant from the primary cultures and also carried for 12 days. Both sets of HeLa cultures were set up in 35-mm-diameter wells, each containing 1.5×10^5 cells in 2 ml of growth medium, the day before infection; every 2 days 1 ml was removed and replaced with 1 ml of fresh medium.

Isolation of revertant, cyclosporin-sensitive viruses from MC1.1G5. Revertant, cyclosporin-sensitive viruses were isolated from HeLa cells infected with the recombinant mutant MC1.1G5. Infection of a culture was established by exposure to 1 μg of SDZ NIM 811 per ml followed by serial passage of the cells in the absence of cyclosporin. When viral replication (assessed by $p24^{gag}$ assays) became apparent, chromosomal DNA was extracted, proviral DNA was amplified, and recombinant G-segment chimeras in the NL4-3 vector (described above) were constructed. Recombinant plasmid clones were transfected for virus recovery, and one replication-competent clone (R8) was found and characterized.

RESULTS

Effect of cyclosporins on the replication of HIV-1 strain NL4-3 in HeLa cells and appearance of cyclosporin-resistant and -dependent viral mutants. Replication of NL4-3 is inhibited by CsA over the course of 12 days in a culture of HeLa cells (Fig. 2A). Between days 6 and 10, during which virus in the absence of the drug replicates most rapidly, a concentration as low as 1.0 μg/ml inhibits the accumulation of $p24^{gag}$ in culture supernatants by at least 90%. Because the cultures reach the monolayer stage by day 8, it was necessary to transfer the virus onto fresh cell cultures in order to monitor replication for longer periods of time. Therefore, supernatants from 6-day primary cultures were used to infect secondary cultures. In the latter (Fig. 2A′), levels of $p24^{gag}$ rapidly increase, and in the presence of CsA (1 and 3 μg/ml), virus replication appears only to follow a 2 to 4-day delay period, ultimately producing levels as high as those in the absence of drug. The influence of CsA on the replication of the virus in these cells may be characterized as a "leaky" inhibition of virus spread at low multiplicities of infection, which dramatically loses antiviral activity at some limiting threshold of infection. In the presence of SDZ NIM 811 (Fig. 2B), the accumulation of $p24^{gag}$ is slower than that seen with CsA, but by day 12 in secondary cultures it has reached the highest level seen in the primary culture in the absence of cyclosporin (Fig. 2B). The characteristic rapid decline in $p24^{gag}$ after the peak value was accompanied by cell

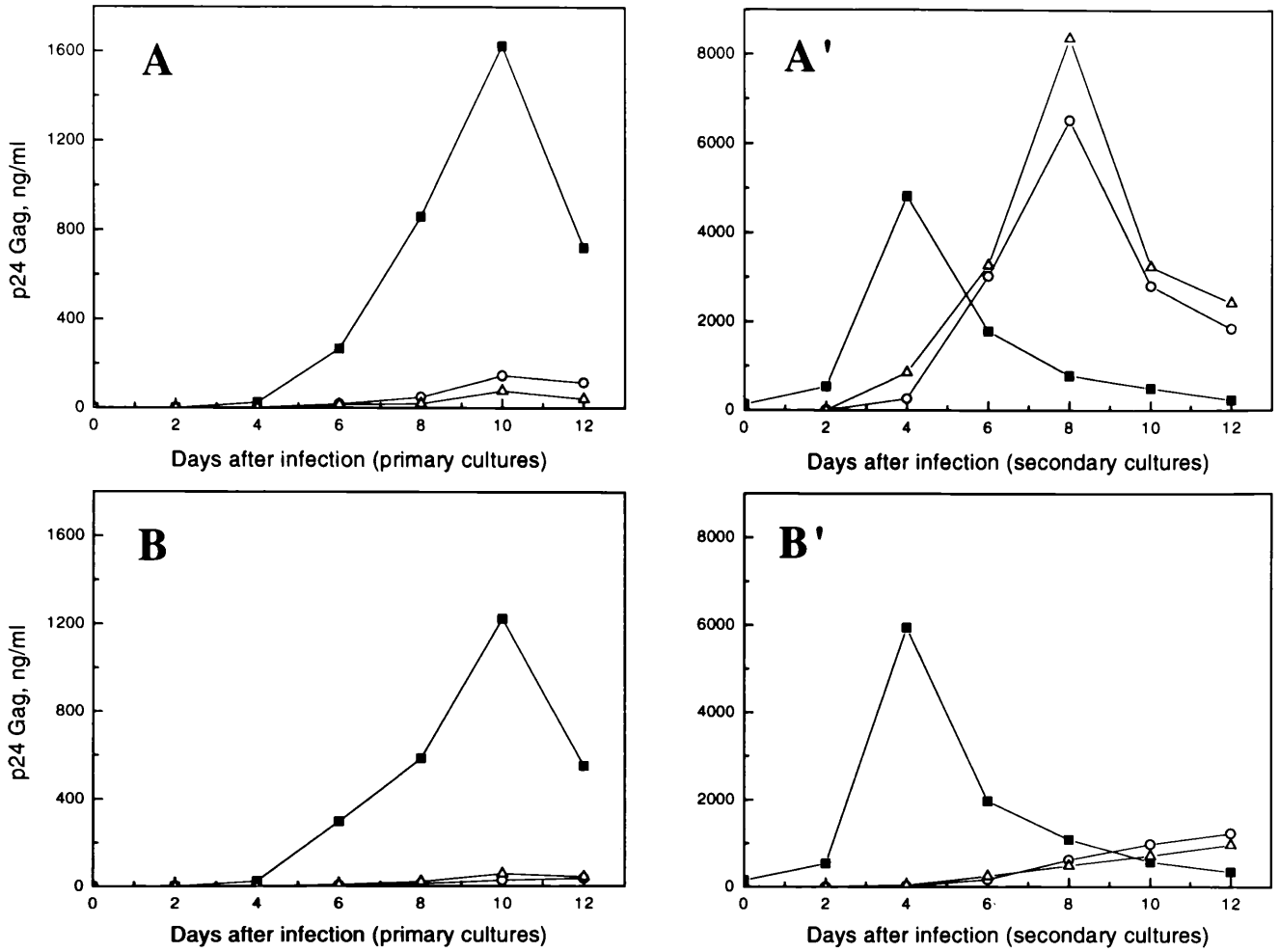

FIG. 2. Kinetic analysis of cyclosporin inhibition of NL4-3 replication in HeLa cells. In order to monitor replication for longer than the time it took for the infected primary cultures (A and B) to form monolayers, 6-day supernatants were transferred to secondary cultures (A′ and B′, respectively). The amount of virus produced from the infected cultures was estimated from $p24^{gag}$ concentrations in the culture supernatants. Culture conditions and assays are described in detail in Materials and Methods. Each point represents the average value from duplicate infected cultures, and all points vary by less than 20% from the respective mean values. The effects of CsA (A and A′) and of SDZ NIM 811 (B and B′) at drug concentrations of 3 μg/ml (○) and 1 μg/ml (△) are shown. ■, no drug.

death in all infected cultures, with or without either cyclosporin. With HeLa cells, in experiments longer than several days, it was not possible to test concentrations of CsA or SDZ NIM 811 higher than 3 μg/ml because of cell toxicity.

The measurable replication of NL4-3 in HeLa cells in the continuous presence of cyclosporins at concentrations as high as 3 μg/ml, during extended time periods by serial transfer of virus into fresh cultures, suggested that one might be able to enrich for spontaneous mutants with relative resistance to inhibition by cyclosporins under these conditions. Indeed, after six serial passages of an NL4-3 stock, followed by endpoint dilution, in the presence of SDZ NIM 811 (see Materials and Methods), cyclosporin-resistant viruses could be isolated. In preliminary tests of replication in response to cyclosporin (SDZ NIM 811 or CsA) concentration, the mutant viruses showed both a dependence on cyclosporin and a resistance at higher concentrations. In total, four independent mutant viruses, named MC1.1, MC1.3, MC2.1, and MC3.2, were isolated and purified twice by endpoint titration.

Analysis of $Cs^{R/D}$ mutants by construction of recombinant proviruses in the NL4-3 background. From HeLa cell cultures infected with the endpoint-diluted mutant viruses, nuclear DNA was extracted and used as template for PCR amplification of selected segments of the proviruses (Fig. 1). Each of these segments was then substituted individually for the homologous region in pNL4-3, creating a set of recombinant proviruses from which viruses were rescued and tested in order to map the $Cs^{R/D}$ mutation(s). From the mutant virus MC1.1, chimeras with NL4-3 were created for all five recombinant segments (G, GP, P, E, and EN), covering nearly the full provirus. As summarized in Table 1, tests of these various chimeras indicate unambiguously that the $Cs^{R/D}$ phenotype maps solely within the G segment; all of the chimeras constructed from other segments of MC1.1 either yield wild-type (cyclosporin-sensitive) virus or fail to yield virus. Tests of G- and GP-segment chimeras from the other three MC mutants give the same results. The replication of G-segment recombinant viruses (one from each of the four MC mutants) in response to various CsA and SDZ NIM 811 concentrations is shown in Fig. 3. Several features common to the mutant G-segment recombinants are evident: (i) no viral replication in the absence, or at low concentrations, of one of the cyclosporins; (ii) significant viral replication at relatively high concentrations of either cyclosporin; and (iii) some inhibitory effects

TABLE 1. Transfection of recombinant mutant–NL4-3 proviruses to test for recovery of the cyclosporin-resistant and -dependent phenotype

Mutant virus	Recombinant segment	No. of clones tested	No. of clones with phenotype:		
			wt[a]	$Cs^{R/D}$	nvr[b]
MC1.1	G	6	0	4	2
	GP	3	3	0	0
	P	12	7	0	5
	E	4	3	0	1
	EN	8	8	0	0
MC1.3	G	5	0	2	3
	GP	5	3	0	2
MC2.1	G	6	0	4	2
	GP	6	3	0	3
MC3.2	G	6	0	3	3
	GP	6	1	0	5

[a] wt, wild-type cyclosporin sensitivity comparable to that of a transfected pNL4-3 control.
[b] nvr, no virus recovered.

at the highest concentration tested (3 μg/ml), although this may be due to cellular cytotoxicity.

Several types of control experiments were included in the analysis of the recombinant proviruses (data not shown). To provide evidence that the mutations found in *gag* G segments were not introduced during PCR or subsequent cloning steps, recombinant G-segment viruses were constructed from MC1.1 proviral DNA amplified in five different PCRs; tests of viruses recovered from eight recombinant clones yielded no wild-type virus, two $Cs^{R/D}$ viruses, and six defective viruses. Similarly, recombinant G-segment viruses were constructed from pNL4-3 DNA amplified in five different PCRs, and of eight recombinant clones tested, seven yielded wild-type viruses, none yielded $Cs^{R/D}$ virus, and one yielded a defective virus. To rule out the possibility that the $Cs^{R/D}$ mutations arose from wild-type virus during the course of transfection of proviral DNA into COS-1 cells or subsequent replication in HeLa cells during phenotype testing, pNL4-3 was always carried through all steps of the experiments along with the chimeric proviruses. This control additionally provided an internal measure of the efficiency of virus recovery and cyclosporin sensitivity for a given experiment. From the results of these different control experiments, we conclude that the mutations conferring the

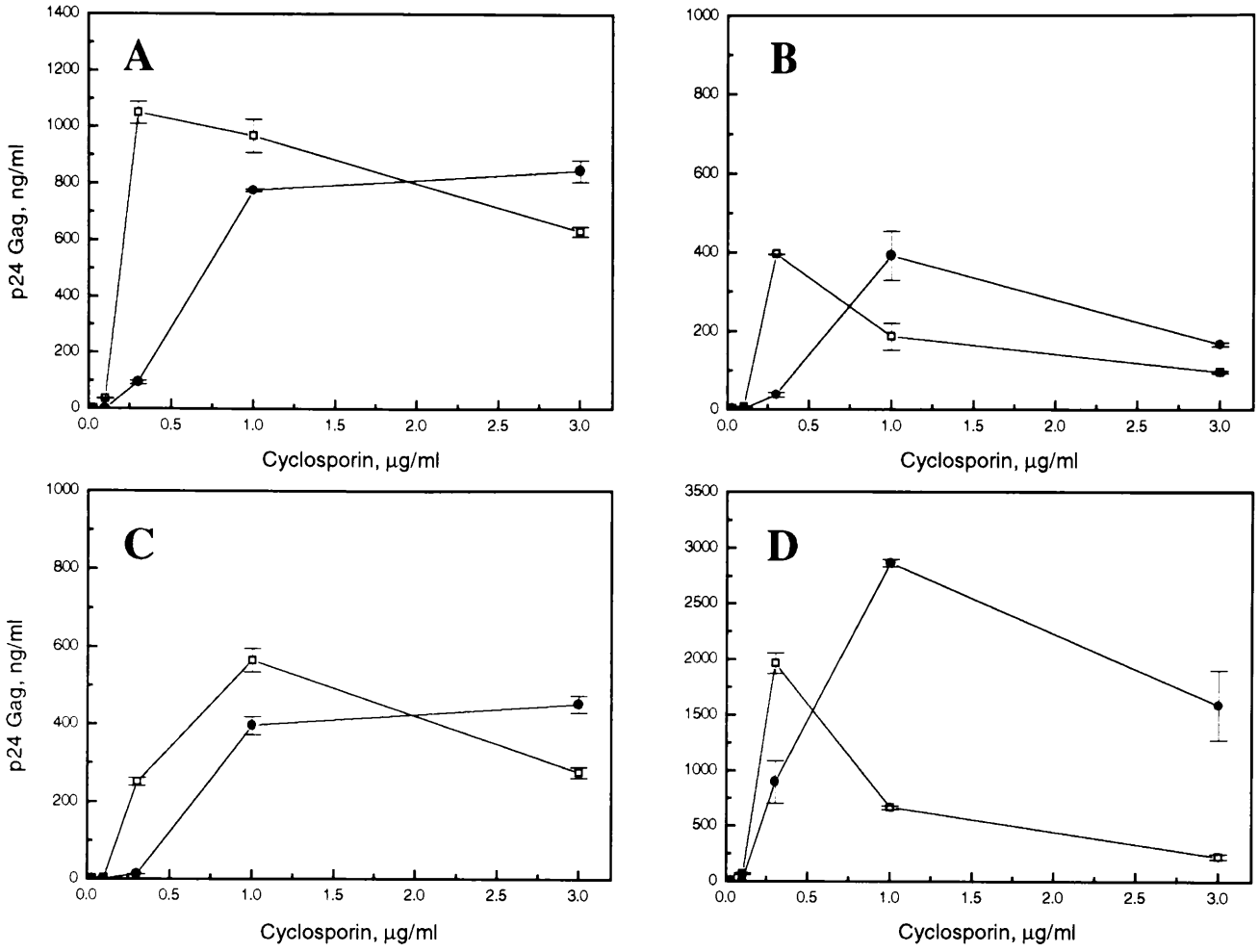

FIG. 3. Replication of mutant G-segment recombinant viruses in HeLa cells is cyclosporin resistant and dependent. The effect of various concentrations of CsA (●) and SDZ NIM 811 (□) on the replication of viruses in 6-day assays in HeLa cells (described in detail in Materials and Methods) was examined. (A) MC1.1G5; (B) MC1.3G4; (C) MC2.1G6; (D) MC3.2G4.

TABLE 2. Mutations in the mutant G segment of recombinant, cyclosporin-resistant and -dependent viruses

Chimeric provirus	Sequence position[a]	Mutation (NL4-3→mutant)[b]
MC1.1G5	1130	AAG (Lys)→A**T**G (Met)
	1323	GGA (Gly)→GG**T** (Gly)
	1460	GCA (Ala)→G**A**A (Glu)
	1613	AGA (Arg)→A**A**A (Lys)
MC1.3G4	1466	GGC (Gly)→G**A**C (Asp)
MC2.1G6	1460	GCA (Ala)→G**A**A (Glu)
MC3.2G4	1466	GGC (Gly)→G**A**C (Asp)
	1476	AGA (Arg)→AG**G** (Arg)
	1707	GCC (Ala)→GC**T** (Ala)

[a] Position, relative to the published NL4-3 proviral sequence (accession no. M19921), of the nucleotide substitution present in the mutant virus.

[b] NL4-3 and corresponding mutant virus *gag* codons in which the base substitution is present (substitutions are in boldface).

$Cs^{R/D}$ phenotype and captured in the G-segment chimeras of all four independent MC mutants are truly mutations that arose spontaneously in NL4-3 during replication in HeLa cells in the presence of SDZ NIM 811.

From many of the proviral recombinant plasmid DNA clones, no virus could be recovered (Table 1; see preceding paragraph), for which there are several possible explanations. Defective proviral DNA may have been amplified by PCR, or the *Taq* polymerase may have introduced deleterious mutations during amplification. Alternatively, there may have been mistakes introduced at a cloning step.

DNA sequence analysis of the mutant *gag* G segments. The DNA sequence of the entire 1,300-bp G segment (between the *Bss*HII and *Apa*I restriction enzyme sites [Fig. 1]) was determined for four different chimeras, one from each of the four original MC mutant isolates. The mutations that were found, relative to the *gag* sequence of NL4-3, are summarized in Table 2. Two of the chimeras (MC1.1G5 and MC3.2G4) contain multiple mutations; however, the other two (MC1.3G4 and MC2.1G6) contain only a single mutation. Of interest was the observation that they all share one of two possible mutations (diagrammed in Fig. 4A) within the capsid domain of $Pr55^{gag}$ that lie on either side of the third of four closely spaced proline residues (Fig. 4B). Both mutations result in a nonconservative amino acid exchange (i.e., alanine to glutamic acid and glycine to aspartic acid). Clone MC1.1G5 has three additional mutations, one of which is silent, and clone MC3.2G4 has two additional silent ones, all of which are well outside the proline-rich amino acid stretch (Fig. 4A).

Cyclosporin dependence of the mutant viruses in HeLa cells in stringent: isolation of revertant virus in the absence of cyclosporins. The replication of MC1.1G5 was examined more closely in a kinetic analysis, as described above for NL4-3 (the NL4-3 results are shown in Fig. 2). In the absence of cyclosporin, MC1.1G5 is unable to replicate in HeLa cells, even after transfer of culture supernatants onto fresh cells for an extended time period, under conditions in which the virus replicates rapidly in the presence of either CsA (Fig. 5A and A′) or SDZ NIM 811 (Fig. 5B and B′). However, when infection of HeLa cell cultures with MC1.1G5 is established in the presence of SDZ NIM 811 and then subsequently cyclosporin is withdrawn and the infected cells are cultivated by serial passage (see Materials and Methods), after several passages detectable levels of $p24^{gag}$ begin to appear in the supernatant.

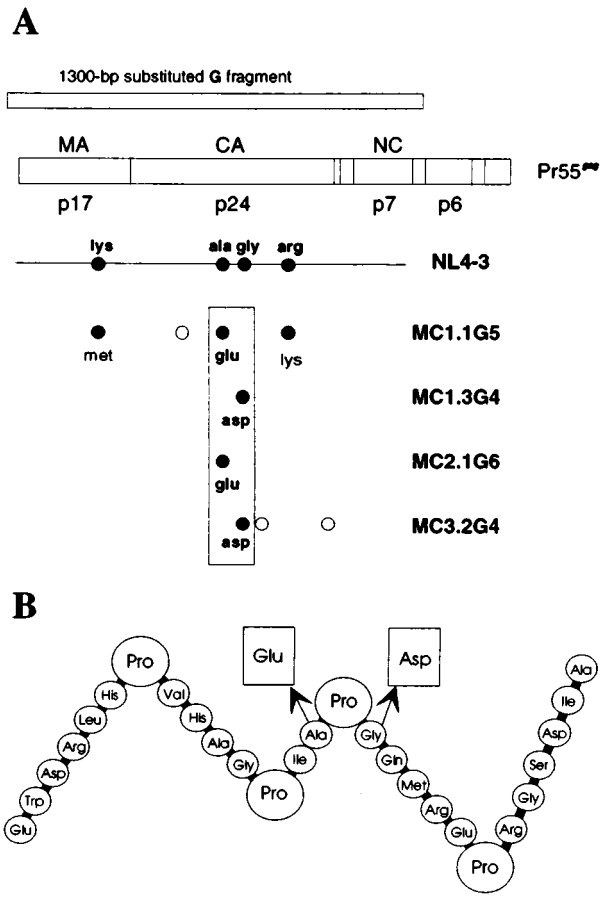

FIG. 4. (A) Sequence analysis of the mutant G segments present in the recombinant proviruses MC1.1G5, MC1.3G4, MC2.1G6, and MC3.2G4. At the top the position of the G fragment relative to *gag* coding sequences is shown, below which all mutations in the individual G segments relative to the parental NL4-3 sequences are indicated. ●, amino acid changes; ○, silent mutations. The two mutations of which at least one is common to all of the mutant sequences are outlined by the rectangular box. The NL4-3 codon affected by each of the mutations is indicated, as is each amino acid exchange in the mutants. (B) The proline-rich CA region in which the two common MC mutations occur. The corresponding amino acid exchanges are indicated by arrows.

From such an experiment, the virus that slowly appeared after 3 weeks was tested for cyclosporin effects, with the result that the virus had reacquired the wild-type, cyclosporin-sensitive replication phenotype. For analysis of the revertant virus, recombinant G-segment proviruses in the NL4-3 vector were constructed as for the MC mutants (except that the revertant virus was not endpoint diluted), and one chimeric provirus, R8, that yielded non-replication-defective virus was obtained from four clones tested. Replication of R8 shows the same response to different concentrations of CsA or SDZ NIM 811 as that of NL4-3 (Fig. 6). DNA sequence analysis of the entire R8 G segment revealed that in addition to all of the mutations in MC1.1G5, two additional mutations were present, each of which results in an amino acid exchange: a mutation of GTG (Val) in MC1.1G5 to GCG (Ala) in R8 at position 1442 (relative to the NL4-3 provirus sequence) and a mutation of GCA (Ala) in MC1.1G5 to ACA (Thr) in R8 at position 1498. Both of the mutations are within the region shown in Fig. 4B.

Replication of the recombinant mutant viruses in CEM cells is also cyclosporin dependent. It was initially observed that the original, endpoint-diluted MC1.1 and all four MC–NL4-3 chi-

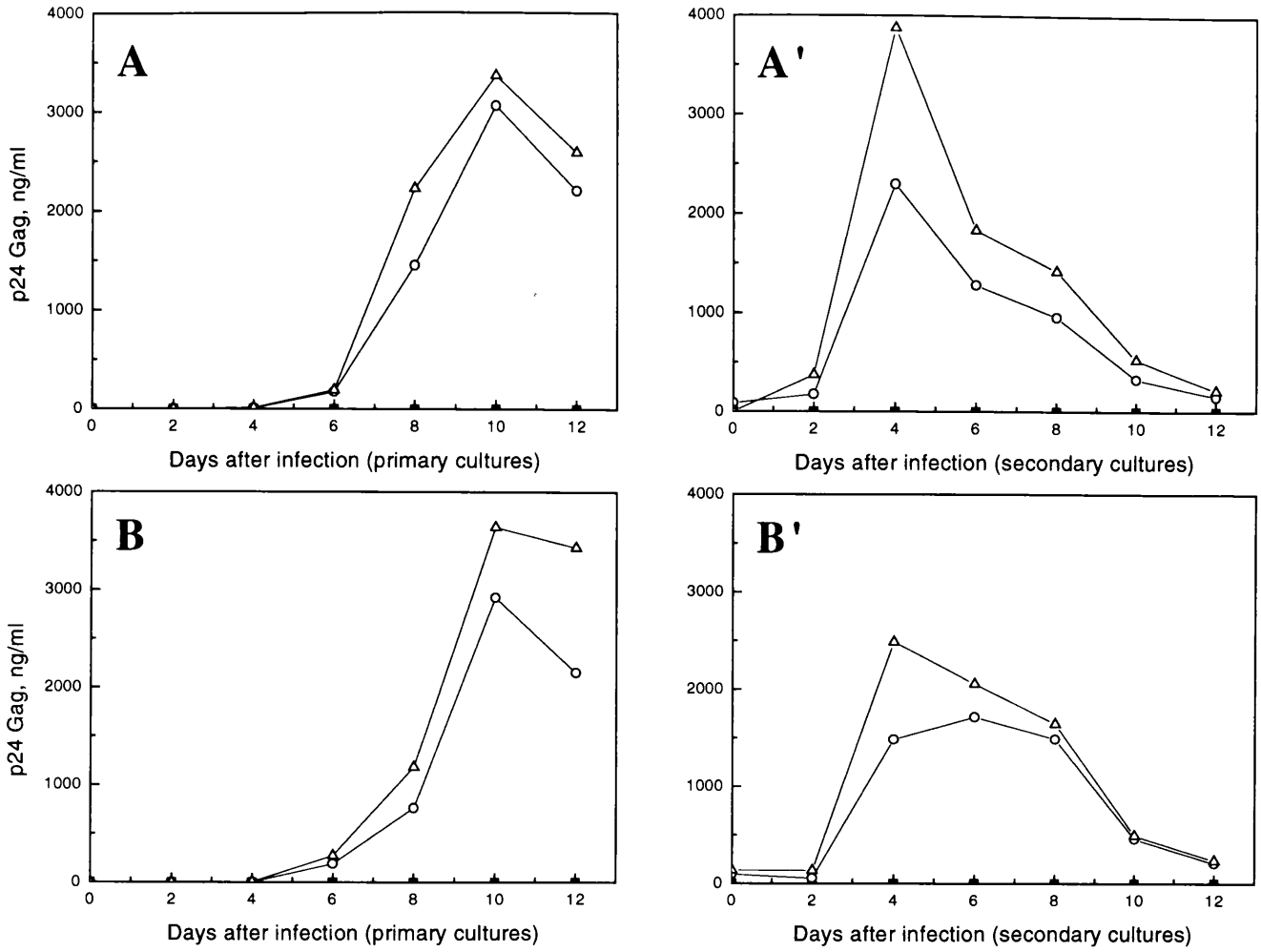

FIG. 5. Kinetic analysis of cyclosporin-dependent replication of MC1.1G5 in HeLa cells. The panels and symbols are as described in the legend to Fig. 2. Each point represents the average value from duplicate infected cultures, and all points vary by less than 14% from the respective mean values.

meras replicated inefficiently in short-term dose-response assays in CEM cells with or without cyclosporin. However, in a kinetic analysis of replication in CEM cells over a 14-day period, under conditions in which NL4-3 peak replication is observed after 8 days (Fig. 7), it is seen that MC1.1G5 does indeed replicate, but only in the presence of cyclosporin (Fig. 8). Even under optimal conditions for replication in CEM cells, i.e., in the presence of CsA or SDZ NIM 811, MC1.1G5 replicates more slowly than NL4-3 (the data presented in Fig. 7 and 8 are from the same experiment). MC1.3G4 and MC2.1G6 were also tested and exhibit cyclosporin-dependent replication (Fig. 9). MC2.1G6 appears to grow poorly in CEM cells (Fig. 9B), relative to MC1.3G4 in the presence of cyclosporin (Fig. 9A) or relative to NL4-3 in the absence of cyclosporin (Fig. 9C) in the same experiment.

DISCUSSION

HIV-1 replicating in HeLa cells spontaneously gives rise to $Cs^{R/D}$ mutants that may be enriched in the presence of the nonimmunosuppressive cyclosporin SDZ NIM 811 and purified by endpoint dilution. In four independently isolated mutants, the mutations conferring the $Cs^{R/D}$ phenotype are located only in *gag* sequences; no other regions analyzed by recombination confer the phenotype. The four isolates share one of two possible mutations within the CA domain, either of which results in a nonconservative amino acid exchange and is sufficient for the $Cs^{R/D}$ phenotype. The mutations occur at either of the residues directly flanking a proline, which is the third of four closely spaced proline residues, within a region already shown by in vitro mutagenesis to be critical for Gag-CyP interaction (8). This proline-rich region is highly conserved in HIV-1 sequences, although a variation has been observed in proviral sequences from the clinical isolate $HIV\text{-}1_{MN}$ (9) at one of the positions of the $Cs^{R/D}$ mutations (N terminal to the third proline). In vitro mutagenesis of the second proline, which is separated from the third proline by only two residues, to an alanine abolishes Gag-CyP interactions in binding assays, incorporation of CyP into virus particles, and infectivity (8). However, there is some flexibility to accommodate mutation in the region, because, in addition to the two conditional $Cs^{R/D}$ mutations, we also observe an apparent suppressor mutation(s) in a revertant isolate derived from one of the $Cs^{R/D}$ viruses.

Originally it was reported that HIV-1 $Pr55^{gag}$ binds to CyP A and B in vitro and that this binding is sensitive to disruption by CsA (13). More recently, there have been other reports that demonstrate Gag-mediated incorporation of CyP A into virus

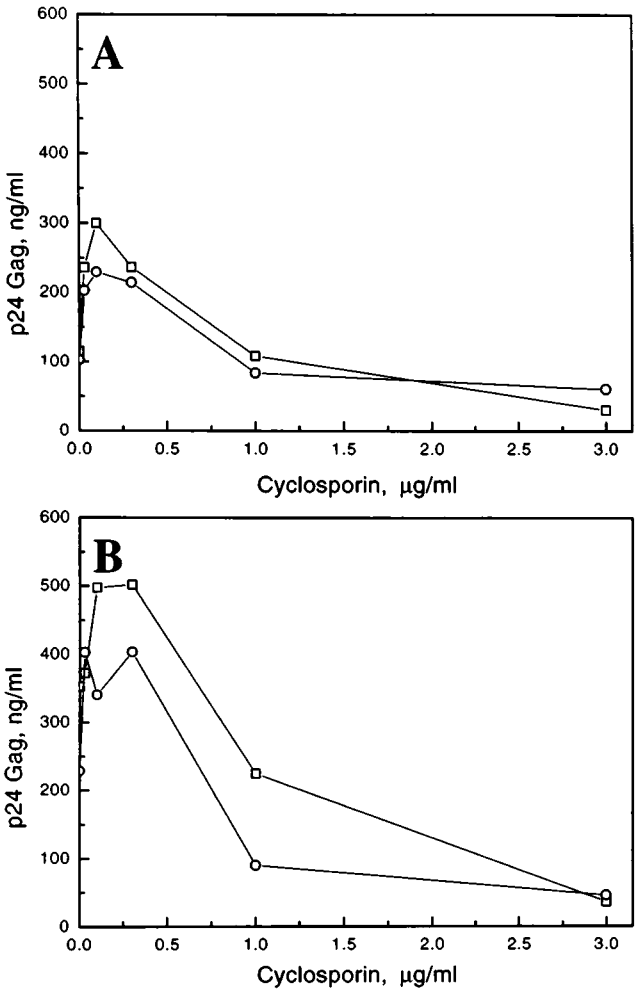

FIG. 6. Comparison of the replication of revertant virus R8 (A) and NL4-3 (B) in HeLa cells. The effect of various concentrations of CsA (○) and SDZ NIM (□) was tested in 6-day assays in HeLa cells (described in detail in Materials and Methods).

particles and suggest that this incorporation is necessary for the production of infectious virions (8, 20). It is not yet clear what effects the $Cs^{R/D}$ mutations may have on Gag-CyP binding or incorporation of CyP into virions, or, for that matter, on biochemical effects on certain steps in the HIV-1 life cycle reported for cyclosporins (18). Preliminary experiments indicate that the properties of in vitro binding of recombinant mutant $Pr55^{gag}$ to CyP A are comparable to those for the wild-type protein and that this binding is as sensitive to disruption by CsA as that of the wild type (12a). This result is unexpected if one assumes that Gag-CyP complex formation is necessary for HIV-1 infectivity and that the disruption of this interaction explains the antiviral activity of cyclosporins. On the contrary, in the simplest model the mutant $Pr55^{gag}$ might be expected to bind CyP only in the presence of cyclosporin, and one would imagine that the $Cs^{R/D}$ mutations in Gag change the structure of the CyP-binding region in such a way that binding would favor CyP-cyclosporin complexes. As a second interpretation, one might conclude that the $Cs^{R/D}$ mutations in Gag cause a subtle increase in affinity for CyP that simply is not evident in an in vitro binding assay but which nonetheless in vivo results in a much more stable complex; the dependence phenomenon may then be explained by the necessity to dissociate the complexes at a second step, and the presence of cyclosporin would facilitate that process. Alternatively, one might speculate that Gag is actually a substrate for the CyP rotamase activity, which has been shown to be inhibited by CsA (6, 7, 19), and that the Gag-CyP complex as such represents an enzyme-substrate complex. Interestingly, this model would fit the in vitro binding data well if one postulates that the $Cs^{R/D}$ mutations in the context of the proline-rich region form a unique secondary structure, necessary for some Gag activity, whose analogous structure in wild-type Gag is formed by the action of rotamase and that the action of rotamase on the mutant protein would actually be to disarrange this secondary structure. One final possibility is that the mutant Gag proteins have now adapted to bind to a second cellular protein, whose function replaces that of CyP.

It was unexpected to obtain only drug-resistant and -dependent mutants of HIV-1 with cyclosporins and to obtain no mutants showing a simple drug resistance. Reports of drug-dependent mutants, either viral (14, 15, 21) or bacterial (3), in the literature also have included the isolation of drug-resistant

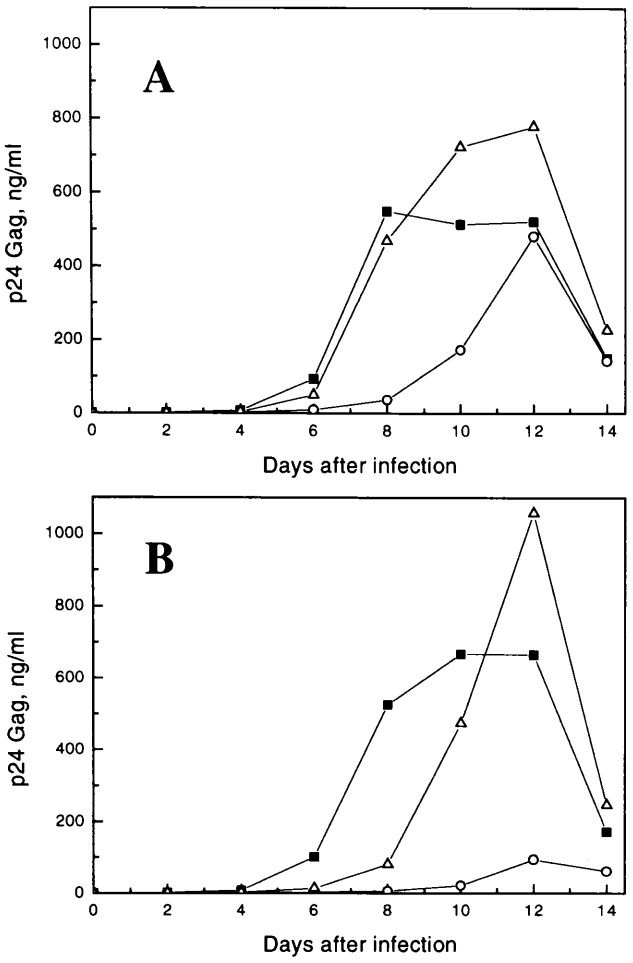

FIG. 7. Kinetic analysis of cyclosporin inhibition of NL4-3 replication in CEM cells. Culture conditions and assays are described in detail in Materials and Methods. The effects of CsA (A) and of SDZ NIM 811 (B) at drug concentrations of 1 μg/ml (○) and 0.3 μg/ml (△) are shown. ■, no drug. Each point represents the average value from duplicate infected cultures, and all points vary by less than 20% from the respective mean values, except for 40% at points corresponding to absence of drug on day 10 (A) and day 8 (B).

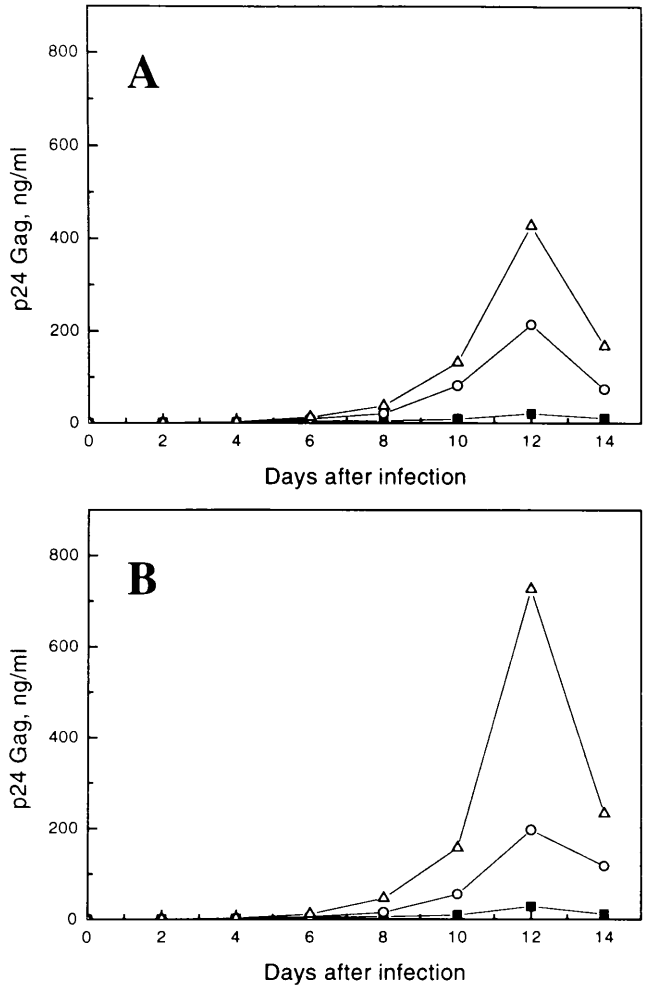

FIG. 8. Kinetic analysis of cyclosporin-dependent replication of MC1.1G5 in CEM cells. The panels and symbols are as described in the legend to Fig. 7. Each point represents the average value from duplicate infected cultures, and all points vary by less than 20% from the respective mean values.

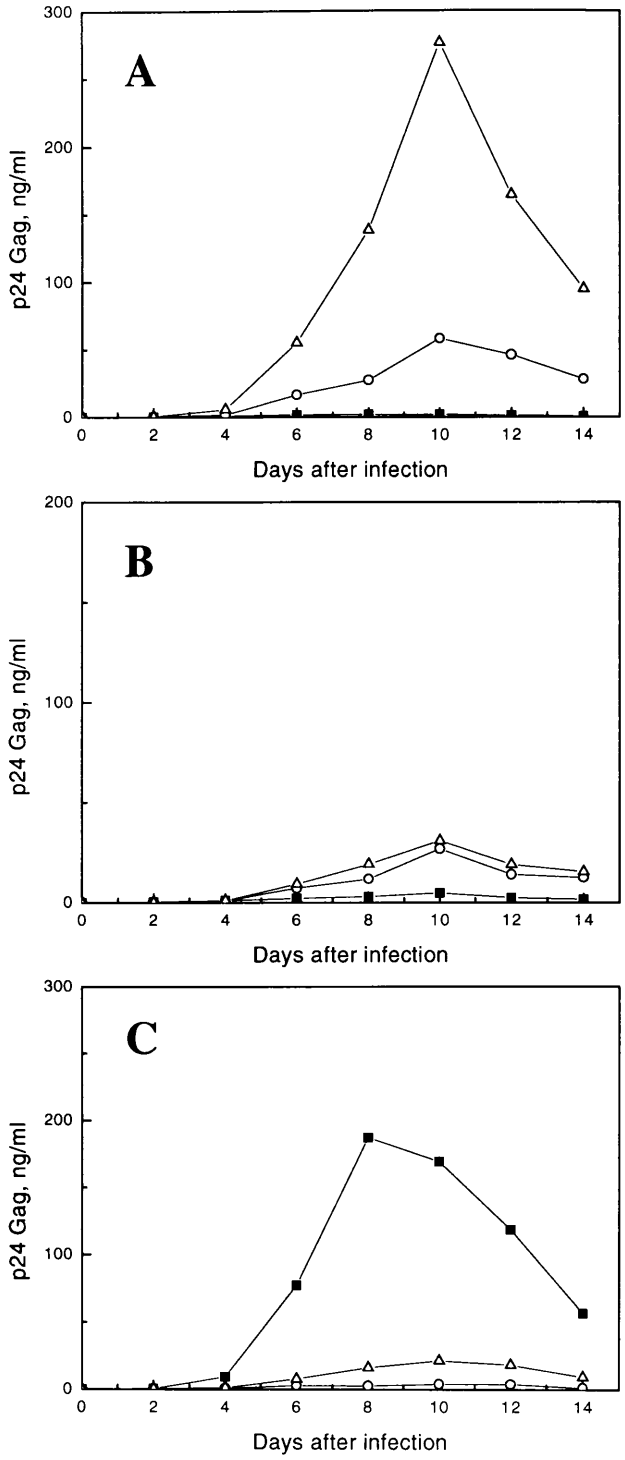

FIG. 9. Effects of cyclosporins on replication in CEM cells of MC1.3G4 (A), MC2.1G6 (B), and NL4-3 (control) (C). Mutant viruses are dependent on CsA or SDZ NIM 811 for replication, following viral replication by $p24^{gag}$ over 14 days in CEM cells. ■, no drug; ○, SDZ NIM 811 at 1 μg/ml; △, CsA at 1 μg/ml. Each point represents the average from duplicate infected cultures, and all points vary by less than 24% from the respective mean values.

mutants. One possibility, of course, is that simple cyclosporin-resistant mutants occur less frequently and that a sufficient number of viral mutants was not collected and analyzed in this study. However, it is also possible that, at least in HeLa cells in which the mutants were enriched, drug dependence is a necessary consequence of the resistant phenotype. In this regard, it may be of interest that in the revertant R8, selected in HeLa cells, the acquisition of cyclosporin independence was also accompanied by cyclosporin sensitivity. An explanation for obtaining only dependent and resistant mutant viruses will have to await further biochemical characterization of the significance of Gag-CyP complexes. Two observations indicate that the cyclosporin-dependent phenotype, at least in HeLa cells, is an absolute dependence as opposed to a leaky phenotype: (i) in the absence of cyclosporin, drug-dependent mutant viruses do not replicate even over extended time periods, and (ii) only revertant, cyclosporin-independent and -sensitive viruses grow out from infected cultures maintained and passaged in the absence of cyclosporin.

Replication of the recombinant mutant viruses in CEM cells is also cyclosporin resistant and dependent, which indicates that this particular phenotype is not peculiar to HeLa cells. It remains to be seen if such mutants spontaneously arise in infected peripheral T cells, but given sufficient rounds of replication, there is no reason a priori to assume that this would not occur. Because the mutations we have observed that confer

resistance-dependence and reversion are simple base substitutions, in theory it should be possible for HIV-1 to readily adapt to replication conditions either in the continual presence or after the removal of cyclosporins. In considering cyclosporins as possible anti-HIV-1 therapeutic agents, these points should be carefully addressed.

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