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A conditionally replicating HIV-1 vector interferes with wild-type HIV-1 replication and spread

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ABSTRACT **Defective-interfering viruses are known to modulate virus pathogenicity. We describe conditionally replicating HIV-1 (crHIV) vectors that interfere with wild-type HIV-1 (wt-HIV) replication and spread. crHIV vectors are defective-interfering HIV genomes that do not encode viral proteins and replicate only in the presence of wt-HIV helper virus. In cells that contain both wt-HIV and crHIV genomes, the latter are shown to have a selective advantage for packaging into progeny virions because they contain ribozymes that cleave wt-HIV RNA but not crHIV RNA. A crHIV vector containing a triple anti-U5 ribozyme significantly interferes with wt-HIV replication and spread. crHIV vectors are also shown to undergo the full viral replicative cycle after complementation with wt-HIV helper-virus. The application of defective interfering crHIV vectors may result in competition with wt-HIVs and decrease pathogenic viral loads *in vivo*.**

Defective-interfering (DI) viruses have been shown to modulate the pathogenicity of virus infections (1–6). DI viruses generally consist of deleted versions of wild-type (wt) viruses that replicate and compete for packaging into virions at the expense of infectious helper-virus (3–7). In HIV infections, defective HIV genomes are known to exist in HIV-infected hosts (8–11), and experimentally produced defective HIVs have been shown to inhibit wt-HIV replication *in vitro* (12–15). DI-HIVs that spread to uninfected cells have been suggested but not described (16, 17). We have developed DI-HIV vectors that compete with wt-HIV genomes for packaging into virions and spread to uninfected cells in the presence of wt-HIV helper-virus. Conditionally replicating HIV (crHIV) vectors are DI-HIVs that do not encode for HIV proteins that block superinfection (18–20), and contain hammerhead ribozymes (21–23) that cleave wt-HIV RNA. We hypothesized that in cells containing both wt-HIV and crHIV genomes, crHIV RNAs would have a selective advantage for packaging into virions because they contain ribozymes that cleave wt-HIV RNA but not crHIV RNA. Such nonpathogenic crHIV particles would be able to infect uninfected CD4 cells and propagate in the presence of wt-HIV helper-virus, interfering with wt-HIV replication and spread. It is possible that the therapeutic application of such vectors *in vivo* may result in a decrease of wt-HIV loads. Thus, in contrast to current gene therapeutic strategies that require delivery of a dominant negative phenotype into each uninfected cell to interfere with HIV infection (16, 24–30), crHIV genomes are mobile and could be directly delivered (e.g., by liposome-mediated delivery; refs. 31–34) into tissues containing HIV-infected cells (Fig. 1*a*). The aim of this study was to determine whether cells containing wt-HIV and crHIV genomes could selectively package crHIV RNAs into progeny virions and whether such crHIVs interfere with wt-HIV replication and spread.

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MATERIALS AND METHODS

***In Vitro* Transcription.** Ribozyme sequences were cloned into the *Xho*I site of pBluescript II KS. The 0.21-kb mutated crHIV U5 *Bgl*II fragment was cut from the crHIV backbone construct and inserted into the *Bam*HI site of pBluescript. Inserts in pBluescript were linearized with *Bss*HII before *in vitro* transcription. The plasmid expressing wt-HIV U5 RNA has been described (35). It was linearized with *Eco*RI before *in vitro* transcription. Radiolabeled U5 HIV RNA and ribozyme RNA were incubated in reaction buffer (40 mM Tris·HCl, pH 7.5/25 mM $MgCl_2$/2 mM Spermidine/10 mM $NaCl_2$), heated to 65°C for 2 min, and then cooled to 37°C for 5 min before the addition of stop buffer solution (95% formamide/20 mM EDTA/0.05% Bromophenol blue/0.05% Xylene Cyanol FF). The products were then resolved by denaturing PAGE and detected by autoradiography. Experiments were performed in a molar excess of ribozyme RNAs (target/ribozyme ratio of 1:2).

Vectors. crHIV vectors were derived from the pNL4-3 molecular clone (36). pNL4-3 was cut with *Pst*I and *Xho*I, and a polylinker containing convenient restriction sites was inserted in place of the *Pst*I and *Xho*I fragment. The 0.86-kb *Bgl*II to *Bam*HI fragment containing the rev responsive element was cloned into a *Bam*HI site present in the polylinker. A ribozyme cassette containing either single or multiple ribozymes was inserted into a polylinker *Sal*I restriction site (crHIV-1.1, crHIV-1.11, crHIV-1.12, and crHIV-1.111). Ribozymes inserted into crHIV constructs contained a 22-base catalytic domain and two 9-base hybridization domains. Complementary deoxyoligonucleotides encoding ribozyme sequences were synthesized, annealed, and then cloned into the *Sal*I site. The ribozymes were targeted to the +115 and +133 sites of HIV U5 RNA and consisted of the following sequences: +115 site, CACACAACACTGATGAGGCCGAAAGGCCGAAACG-GGCACA; and +133 site, ATCTCTAGTCTGATGAGGCC-GAAAGGCCGAAACCAGAGTC.

Cells and Transfection. Jurkat cells (10^6) were washed in Opti-MEM medium (Life Technologies, Grand Island, NY) and then cotransfected with wt-HIV DNA (pNL4-3; 0.6 μg) and crHIV-1.11 DNA (1.8 μg). DNA was mixed in lipofectamine solution (Life Technologies) for 30 min and then incubated on Jurkat cells for 3–6 hr, after which complete RPMI medium containing 10% fetal bovine serum (FBS) and 20 mM Hepes was added. A molar ratio of wt-HIV to crHIV of 1:3 was used to ensure that cells transfected with wt-HIV proviruses also contained crHIV DNA. Virus-containing supernatants were harvested every 2–4 days, and virus assayed by reverse transcriptase activity. For the transfection into ACH2 cells, 10^6 ACH2 cells were transfected with 2.5 μg of vector DNA, after which they were stimulated with 50 ng/ml of 12-*O*-tetradecanoylphorbol 13-acetate 24 hr after transfection. RNA was isolated from cell supernatants 48 hr later and analyzed by reverse transcription

Abbreviations: cr, conditionally replicating, wt, wild type, DI, defective-interfering; RT, reverse transcription.

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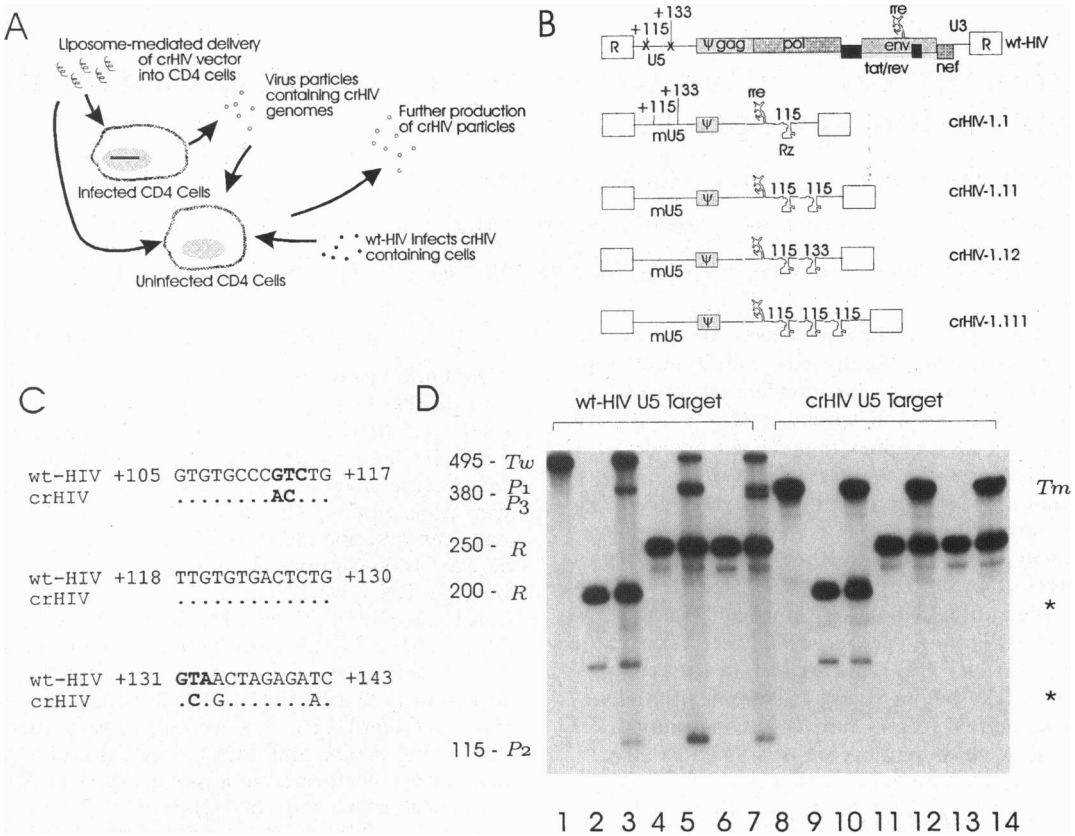

FIG. 1. crHIV vector strategy and design. (*A*) The crHIV vector strategy showing how crHIV DNA is initially delivered (e.g., by liposome-mediated delivery) into either infected or uninfected CD4 cells. Infected cells containing crHIV genomes selectively produce crHIV RNA-containing particles that can infect uninfected cells. Uninfected cells containing crHIV genomes can be superinfected with wt-HIV that results in further crHIV particle production. (*B*) Structure of crHIV vectors containing either single or multiple ribozymes. (*C*) wt-HIV U5 RNA and crHIV U5 RNA sequences. The crHIV U5 RNA was made resistant to cis ribozyme cleavage by substituting an oligodeoxynucleotide containing the depicted base modifications into the U5 region of crHIV DNA. Numbers refer to the number of nucleotides downstream from the start of transcription. (*D*) Susceptibility of wt-HIV U5 RNAs and resistance of crHIV U5 RNAs to *in vitro* ribozyme cleavage. The wt-HIV U5 RNA (lane 1; Tw, 495 bases) and the crHIV U5 RNA (lane 8; Tm, 374 bases) transcripts are shown. Lanes 2 and 9 show ribozyme RNA that contains a single ribozyme (R, 198 bases) targeted to the +115 site. Lanes 4 and 11 show ribozyme RNA that contains a double ribozyme (R, 238 bases) targeted to +115. Lanes 6 and 13 show ribozyme RNA that contains a double ribozyme (R, 238 bases) targeted to sites +115 and +133. Lane 3 shows cleavage of wt-HIV U5 RNA with the single ribozyme that results in products P1 and P2. Increased cleavage of wt-U5 HIV RNA occurs after incubation with a double ribozyme targeted to same site +115 (lane 5). When a ribozyme RNA to two different sites were incubated with wt-U5 HIV RNA, products P1 (380 bases), P2 (115 bases), and P3 (362 bases) were seen (lane 7). P3 results from cleavage at the +133 site. In contrast, crHIV U5 RNA showed no cleavage when incubated with either of the above ribozyme RNAs (lanes 10, 12, and 14). The asterisks show where the cleavage products would have resolved.

(RT)-PCR. To examine whether crHIV containing virions from the above supernatants could infect uninfected Jurkat cells, 10^6 Jurkat cells were incubated with the RT normalized supernatants for 2 hr at 37°C and analyzed for crHIV DNA 24 hr later. For experiments showing susceptibility of crHIV-containing Jurkat cells to wt-HIV, 10^6 Jurkat cells were transfected with 2.5 μg of crHIV DNA. The cells were allowed to grow for 48 hr before infection with wt-HIV stock virus. crHIV-1.11-transfected Jurkat cells were incubated with stock NL4-3 HIV (2×10^5 tissue culture 50% infective dose units per 10^6 cells) for 2 hr at 37°C, washed three times in Opti-MEM, and then resuspended in complete RPMI medium (with 10% FBS and 20 mM Hepes). RNA was isolated from cell supernatants 5 days after infection and analyzed by RT-PCR. crHIV vectors that do not contain ribozymes or contain a nonfunctional ribozyme were used in separate control experiments. The nonbase-paired guanosines in the catalytic domain were mutated to adenosines, resulting in ribozymes that do not cleave their targets (37, 38).

Isolation and Detection of HIV RNA. Virion and intracellular viral RNAs were isolated using Trizol (Life Technologies). Intracellular viral RNAs were isolated directly from microcentrifuged cell pellets. Virion RNAs were isolated from cell-free culture supernatants that were first cleared of cells and debris by microcentrifugation at 12,000 × *g* for 5 min. Trizol was added to the cell-free supernatants or cell pellets and incubated for 5 min before the addition of choloroform for phase separation. The aqueous phase was transfered to a fresh tube and the RNA precipitated with isopropanol using glycogen. After reconstitution, the viral RNAs were reverse transcribed and amplified by PCR using radiolabeled primers (see Fig. 3*a*). RT was performed for 1 hr at 42°C in first strand buffer (50 mM Tris·HCl, pH 8.3/75 mM KCl/3 mM $MgCl_2$/5 mM DTT/1 mM dNTPs/20 units of RNase inhibitor) and 25 units of superscript II reverse trancriptase (Life Technologies). After RT was completed, the RT enzyme was heat-inactivated at 65°C for 10 min. The entire mixture was then added directly to a PCR buffer mix (final: 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$) and amplified for 30 cycles using 2.5 units of *Taq* enzyme. The radiolabeled PCR products were then resolved by denaturing PAGE and detected by autoradiography. Anti-U5 HIV ribozyme RNA was detected using the R1 (5′-TGTGACGTCGACCACACAACACTGATG-3′) and R2 (5′-TGTGACGTCGACTCTAGATGTGCCCGTTTCGGC-3′) primers (with a *Sal*I restriction enzyme sites). In crHIV-1.111-expressing cells, single, double, and triple ribozyme amplification products are seen. V1 (5′-GGTTAAGCTTGAATTAGCCCTTCCAGTCCCC-3′) and V2 (5′-GGTTGGATCCGGGTGGCAAGTGGTCAAAAAG-3′) primer set

(with *Bam*HI or *Hin*dIII restriction enzyme sites) amplifies wt-HIV RNAs while the V1 and V3 (5′-CGGATCCACGC-GTGTCGACGAGCTCCCATGGTGATCAG-3′) primer (with *Bam*HI and other restriction enzyme sites) set amplifies crHIV RNAs from a crHIV specific polylinker sequence. The Northern probe was isolated from a 0.21-kb *Bgl*II fragment from the U5 region of pNL4-3 (36).

Isolation and Detection of HIV DNA. DNA was isolated from the organic phase and precipitated with 100% ethanol before washing with 0.1 M sodium citrate in 10% ethanol. After drying, the pellet was solubilized in 8 mM NaOH and then neutralized to a pH of 7.5 with 1 M Hepes before restriction enzyme digestion with *Eco*RI, and then ribozyme DNA was amplified by PCR using R1 and R2 set of primers.

Isolation and Detection of HIV Proteins. Intracellular viral proteins were isolated from transfected cells using Trizol. After addition of Trizol to cell pellets, phase separation of RNA and precipitation of DNA with ethanol, the proteins in the supernatant were precipitated with isopropanol. The protein pellet was then washed by 0.3 M guanidine hydrochloride solution in 95% ethanol, dried, and solubilized in 1% SDS before PAGE. Cellular proteins (3 μg) were resolved by PAGE and electroblotted onto nitrocellulose. HIV Gag proteins were detected by using anti-HIV human antiserum and visualized by chemiluminesence (Amersham) and autoradiography.

RESULTS

Design of crHIV Vectors. crHIV-1 vectors contain sequences required for RNA transcription, tRNA primer binding, dimerization, and packaging (39, 40) and a hammerhead ribozyme cassette (Fig. 1*b*; refs. 22 and 23) that cleaves sequences in the U5 region of wt-HIV RNA but not in crHIV U5 RNA. The U5 region of crHIV RNA was engineered to be resistant to ribozyme scission by substituting critical nucleotides near the cleavage site and in the hybridization domains (Fig. 1*c*). The U to C substitution at sites +114 and +132 in crHIV U5 RNA prevents its scission by ribozymes (38). These base substitutions are present in various other strains of HIV (41), thus minimizing the possibility that these substitutions would decrease the replicative capacity of crHIV-1 genomes. The ribozyme cassette consists of a single, double, or triple ribozymes placed in tandem. Constructs containing either single or multiple (crHIV-1.1, crHIV-1.11, and crHIV-1.111) ribozymes were targeted to the same site, at position +115 (Fig. 1*b*). A construct containing a double ribozyme (crHIV-1.12) was targeted to two different sites of the wt-HIV U5 RNA (Fig. 1*b*; positions +115 and +133).

wt-HIV RNAs but Not crHIV RNAs Are Susceptible to Anti-U5 Ribozyme Cleavage. The ability of anti-U5 HIV ribozymes to cleave wt-HIV U5 RNAs and not crHIV U5 RNAs was tested by mixing radiolabeled ribozyme and target RNAs after *in vitro* transcription. Cleavage products were detected when wt-HIV U5 RNAs were incubated with a transcript containing either a single or a double ribozyme targeted to the +115 site (Fig. 1*d*; lanes 3 and 5). Scission also occurred when wt-HIV U5 RNAs were incubated with RNAs containing double ribozymes targeted to two different sites (Fig. 1*d*; lane 7). The double ribozyme transcripts cleaved wt-HIV U5 RNAs more efficiently than the single ribozyme transcripts (Fig. 1*d*; lanes 3, 5, and 7). In contrast, when the modified U5-crHIV RNAs were incubated with either single or double ribozyme RNAs, no cleavage products were detected (Fig. 1*d*; lanes 10, 12, and 14). These results show that crHIV U5 RNAs are resistant to ribozyme scission, whereas wt-HIV U5 RNAs are effectively cleaved by anti-U5 ribozymes.

crHIV Vectors Inhibit wt-HIV Replication. The ability of crHIV vectors to inhibit wt-HIV replication in infected cells was tested by cationic lipid-mediated cotransfection of wt-HIV proviral DNA and crHIV DNA into Jurkat cells. Virus replication was measured by assaying for reverse transcriptase activity in cell-free culture supernatants. Cotransfection of wt-HIV and crHIV-1.1 DNA into Jurkat cells resulted in temporary inhibition of viral replication as compared with cotransfection of wt-HIV and control (pGEM-3Z) DNA (Fig. 2*a*). Since we have shown previously that anti-U5 ribozymes can effectively inhibit HIV replication intracellularly under colocalized conditions (35), we genetically tested the nature of escape virus growth by cotransfecting wt-HIV and crHIV vector DNA that contains double ribozymes. Cells cotransfected with wt-HIV provirus and double ribozyme-containing crHIV-1.11 or crHIV-1.12 vector DNA (Fig. 1*c*) showed delayed kinetics of escape virus growth when compared with cells cotransfected with crHIV-1.1 vector DNA that contains the single ribozyme (Fig. 2*a*). Furthermore, no difference in the kinetics of escape virus growth was found in cell cultures cotransfected with crHIV-1.11 or crHIV-1.12 DNA (Fig. 2*a*). These data suggested that the appearance of escape virus growth was not the result of either poor association of crHIV RNAs with wt-HIV RNAs or reverse transcriptase-mediated mutations in HIV RNAs, but was rather due to a functional limitation of ribozymes within cells. If poor association between wt-HIV and crHIV RNAs was solely responsible for escape virus growth, then the rate of escape virus growth between single and double ribozyme crHIV cultures would be similar. Alternatively, if escape virus growth resulted from mutated HIV RNA sequences due to reverse transcriptase infidelity, then crHIV-1.12 cultures would show a longer delay

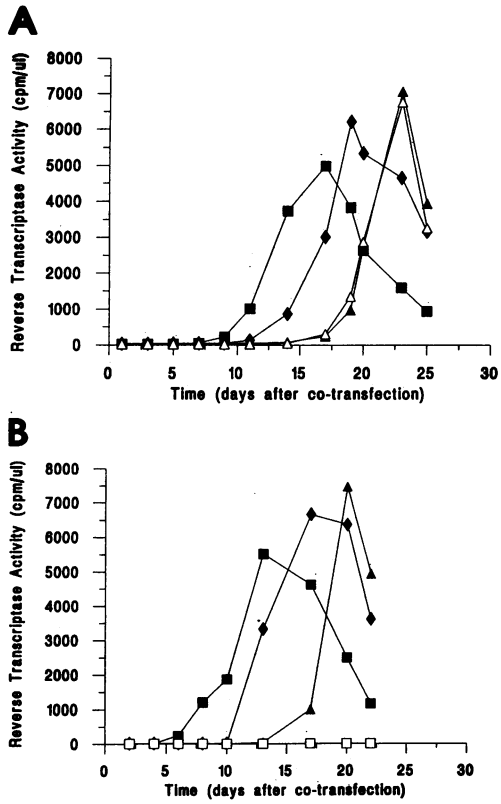

FIG. 2. crHIV-mediated inhibition of wt-HIV replication. (*A*) Inhibition of wt-HIV growth after cotransfection with single and double ribozyme crHIVs. Jurkat cells were cotransfected with wt-HIV and crHIV-1.1 (◆), crHIV-1.11 (▲), crHIV-1.12 (△), or with control pGEM-3Z plasmid (■). (*B*) Further inhibition of wt-HIV replication after cotransfection with a triple ribozyme crHIV. In separate experiments, Jurkat cells were cotransfected with wt-HIV and crHIV-1.1 (◆), crHIV-1.11 (▲), crHIV-1.111 (□) or with control pGEM-3Z plasmid (■). The experiments were done at least three times in duplicate. The higher RT values in crHIV cotransfected cell cultures is a result of increased numbers of cells being present in these cultures when escape virus growth occurs.

in the appearance of escape virus growth than crHIV-1.11 cultures. However, since the kinetics of escape virus growth of crHIV-1.11- and crHIV-1.12-containing cultures were similar, it appears that ribozymes are functionally limited *in vivo* and that multiple ribozymes can relieve this kinetic limitation.

To determine whether further inhibition of wt-HIV replication could be attained, we constructed a crHIV vector that contained a triple ribozyme targeted to the +115 site (crHIV-1.111; Fig. 1*b*). Cotransfection of wt-HIV and crHIV-1.111 genomes produced an additional inhibition of virus growth. Only very low levels of HIV-1 replication could be detected in the supernatants of cells cotransfected with wt-HIV provirus and crHIV-1.111 DNA for up to 22 days in culture (Fig. 2*b*). These data indicate that crHIV vectors that contain multiple ribozymes are very effective inhibitors of wt-HIV replication. crHIV vectors that do not contain a ribozyme or contain a nonfunctional mutated ribozyme did not inhibit wt-HIV replication (data not shown). This result is in agreement with our previous findings, which show that HIVs containing noncleaving mutated ribozymes do not significantly inhibit HIV replication (37).

Mechanism of crHIV-Mediated Inhibition of wt-HIV Replication. To determine the mechanism of crHIV-mediated inhibition of HIV replication, we first examined, by RT-PCR, whether virion-associated ribozyme RNAs were present in cell-free supernatants of cultures cotransfected with wt-HIV and crHIV-1.111 (Fig. 3*a*). The results in Fig. 3*b* show that crHIV-1.111 ribozyme RNAs were present in virions isolated from cell-free supernatants 20 days after cotransfection with wt-HIV-1 and crHIV-1.111 DNA. Thus, crHIV RNA-containing virions were produced from cells displaying very low levels of reverse transcriptase activity, suggesting that crHIV RNAs inhibited wt-HIV replication and spread. The mechanism by which crHIV-1.111 inhibited wt-HIV spread was further examined by comparing intracellular and virion-associated viral RNAs. crHIV-1.111 RNAs were exclusively present within virions, whereas both wt-HIV and crHIV RNAs were detected intracellularly (Fig. 3*c*). This result suggested that crHIV-1.111 RNAs inhibited wt-HIV spread by preferentially cleaving genomic wt-HIV RNAs, resulting in selective packaging of crHIV RNAs into progeny virions. If genomic wt-HIV RNAs are preferentially cleaved, then we should see a decrease of genomic wt-HIV RNAs in 20 day wt-HIV and crHIV-1.111 cotransfected cells. Northern blot analysis of these cells showed almost no genomic wt-HIV RNAs but an abundance of lower molecular weight HIV RNAs (Fig. 4*a*; lane 2). In contrast, control wt-HIV-transfected cells expressed both genomic and subgenomic HIV RNAs (Fig. 4*a*; lane 1). These data are consistent with the suggestion that genomic wt-HIV RNAs are preferentially cleaved by ribozymes within crHIV RNAs. To prove that some genomic wt-HIV RNAs are translated into structural proteins, which is required for virion assembly, we examined whether HIV structural proteins were present in 20-day wt-HIV and crHIV-1.111 cotransfected cells by Western blot analysis. Low levels of the p55 Gag precursor protein, which is translated from genomic wt-HIV RNA, was detected in these cells (Fig. 4*b*). Thus, although most genomic wt-HIV RNAs are cleaved by crHIV RNA, some genomic wt-HIV RNAs are nevertheless translated into proteins for virion production.

Mechanism of crHIV Vector Replication and Spread. The ability of crHIV vectors to undergo the complete viral replicative cycle in the presence of wt-HIV helper was investigated. First, we determined whether lipid-mediated transfection of HIV-1-infected ACH2 cells with crHIV DNA resulted in production of viral particles containing crHIV RNA. As shown in Fig. 5*a*, crHIV ribozyme RNAs were detected by RT-PCR analysis in virions produced by ACH2 cells that were first transfected with crHIV-1.11 DNA and then stimulated with 12-*O*-tetradecanoylphorbol 13-acetate to produce virus. Next, we examined whether crHIV particles derived from the above cultures could infect uninfected Jurkat cells and produce

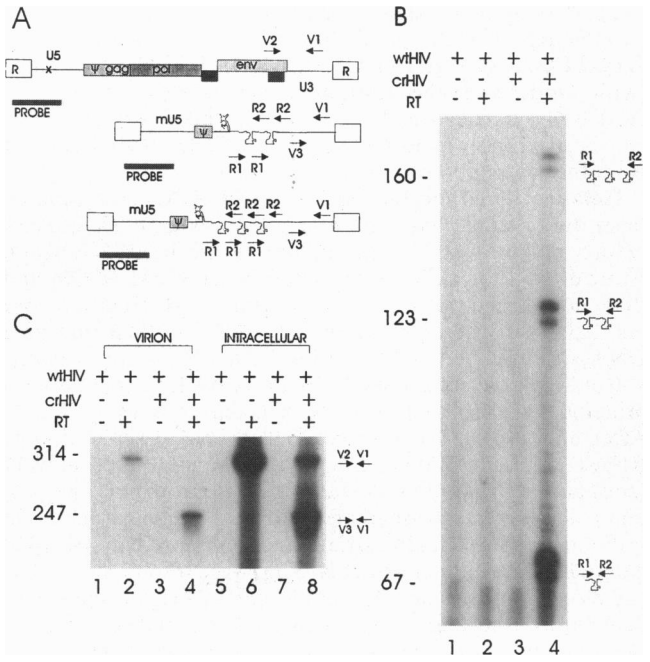

FIG. 3. Analysis of wt-HIV and crHIV RNAs. (*A*) Diagram showing the primers and probe used to detect HIV RNAs. (*B*) RT-PCR amplification of ribozyme RNAs from virions produced by Jurkat cells 20 days post cotransfection with wt-HIV and crHIV-1.111 DNA using R1 and R2 primers. The single, double, and triple ribozyme RT-PCR products are seen in lane 4, while no amplification of ribozyme RNA could be seen in virions produced 20 days after cotransfection of the wt-HIV provirus with the mock control (pGEM-3Z) plasmid (lane 2). Lanes 1 and 3 are RT-PCR negative controls showing that the amplification results from viral RNA alone. (*C*) Amplification of virion (lanes 1–4) and intracellular (lanes 5–8) viral RNAs from cotransfected Jurkat cells 20 days posttransfection using V1, V2, and V3 primers. In crHIV-1.111 and wt-HIV cotransfected cultures, crHIV-1.111 RNAs were exclusively packaged into progeny virions (lane 4). Control wt-HIV and mock cotransfected cultures produced virions containing only wt-HIV RNAs (lane 2). In contrast, both wt-HIV and crHIV-1.111 RNAs were detected intracellularly from crHIV-1.111 and wt-HIV cotransfected cells (lane 8). Only wt-HIV RNAs were observed intracellularly from control wt-HIV and mock-cotransfected cultures (lane 6). Lanes 1, 3, 5, and 7 are RT-PCR negative controls showing that the amplification results from viral RNA alone.

crHIV DNA. Fig. 5*b* shows the presence of crHIV DNA in Jurkat cells infected with cell-free supernatants derived from crHIV-1.11-transfected ACH2 cells (Fig. 5*b*). These results indicated that virions containing crHIV-1.11 RNA were able to infect uninfected T-cells. Since crHIV vectors can spread only in the presence of wt-HIV, we determined whether crHIV particles could be rescued from Jurkat cells containing crHIV genomes after superinfection with wt-HIV. Jurkat cells were first transfected with crHIV DNA and then 48 hr later superinfected with wt-HIV. Virion-associated RNAs from cell-free supernatants were analyzed by RT-PCR 5 days after infection. The results in Fig. 5*c* demonstrate the presence of crHIV RNA-containing virus in cell-free supernatants of Jurkat cells superinfected with wt-HIV. Therefore, *in toto*, these data show that crHIV vectors undergo the full viral replicative cycle after complementation with wt-HIV.

Nature of Escape Virus Growth. The nature of escape virus growth from cultures cotransfected with wt-HIV and crHIV-1.11 was examined by analyzing virion-associated RNAs by RT-PCR using wt-HIV- and crHIV-specific primers (Fig. 3*a*). These results showed that viruses produced by cotransfected cultures at the early stages (Fig. 2*a*; wt-HIV, day +11, and crHIV-1.11, day +19) of virus growth contained predominantly crHIV RNAs. In contrast, viruses produced by cotrans-

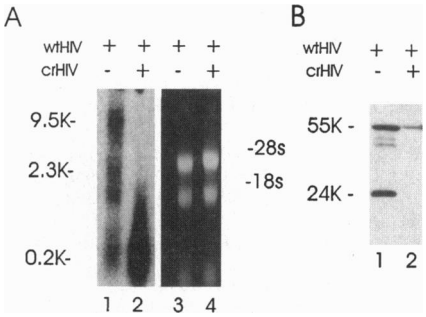

FIG. 4. Analysis of intracellular HIV RNAs and proteins. (*A*) Northern blot analysis of intracellular HIV RNAs from wt-HIV and crHIV-1.111 cotransfected cultures 20 days posttransfection. In wt-HIV cultures all wt-HIV RNA species were detected (lane 1), while crHIV-1.111 cotransfected cultures contain only low levels of genomic (9.7kb) wt-HIV RNA (lane 2). The smearing of wt-HIV RNA from wt-HIV control cells (lane 1) is probably due to RNA degradation that occurs in the population of dead cells present in day 20 wt-HIV cultures (Fig. 3*b*). The low molecular weight HIV RNA smearing from crHIV-1.111 cotransfected cells (that do not show significant numbers of dead cells) is consistent with the suggestion that these HIV RNAs are a mixture of sub-genomic wt-HIV RNAs, crHIV RNAs and cleaved genomic wt-HIV RNAs that are undergoing degradation. The quality of the RNA preparations and the amount loaded is shown in lane 3 (wt-HIV RNA) and lane 4 (crHIV RNA). 10 μg of RNA was loaded and a probe corresponding to the U5 region of HIV was used for detection (probe; Fig. 3*a*). (*B*) Western blot analysis of proteins isolated from wt-HIV and crHIV cotransfected cultures 20 days posttransfection. Abundant production of wt-HIV Gag proteins in wt-HIV cells (lane 1), while crHIV-1.111 cotransfected cells showed low levels of wt-HIV Gag proteins (lane 2).

fected cultures at late stages (Fig. 2*a*; wt-HIV, day +17, and crHIV-1.11, day +23) of virus growth contained predominately wt-HIV RNAs (Fig. 6). Therefore, escape virus growth from cells cotransfected with wt-HIV and crHIV-1.11 resulted from the growth of wt-HIV that escaped from intracellular ribozyme cleavage and spread through the culture. Notably, even at late stages of virus growth crHIV genomes still comprised a significant proportion of the total HIV genomes in virions (Fig. 6). Thus, although wt-HIV genomes predominated at late stages of virus growth, crHIV genomes appeared to nevertheless spread through the culture, albeit at lower efficiencies than wt-HIV genomes.

DISCUSSION

In this study, we describe the development of DI-crHIV genomes that inhibit wt-HIV replication and spread. We demonstrate that crHIV genomes can be conferred with a selective advantage for packaging into virions when they contain ribozymes that cleave wt-HIV RNA but not crHIV RNA. We have also shown that crHIV genomes can spread to uninfected cells after complementation with wt-HIV helper-virus.

Ribozymes were used to provide crHIV RNAs with a selective advantage because they have a high degree of specificity, as determined by their hybridization to complementary target sequences (21–23) and because their efficiency of cleavage depends upon colocalization with target RNAs (35, 42). In HIV-infected cells, colocalization occurs between genomic HIV RNAs when they package and/or dimerize during encapsidation. Therefore, we hypothesized that in cells containing both wt-HIV and crHIV genomes, colocalization of crHIV RNAs with genomic wt-HIV RNAs, and hence cleavage, would occur most efficiently during packaging/dimerization. Conversely, the scission of wt-HIV RNAs that do not efficiently colocalize with crHIV RNAs, such as subgenomic wt-HIV RNAs, would occur less efficiently, allowing for some of these RNAs to be translated into proteins. Our studies demonstrated that cultures cotransfected with wt-HIV and crHIV-1.111 DNA produced virions that exclusively

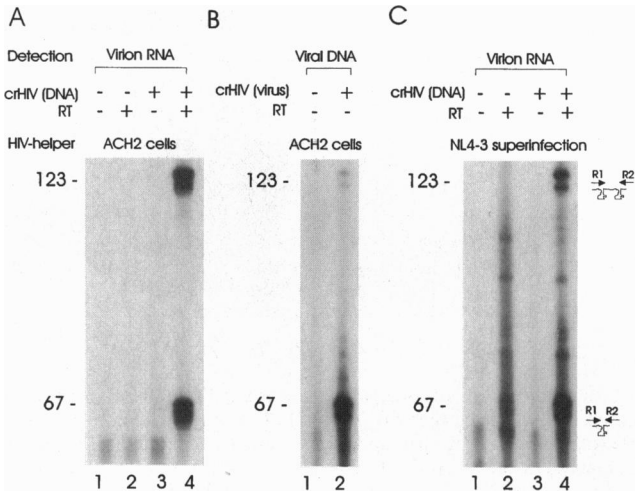

FIG. 5. Replication of crHIV vectors in the presence of wt-HIV helper-virus. (*A*) Production of virions containing crHIV-1.11 RNAs from ACH2 cells transfected with crHIV-1.11 DNA and then stimulated with 12-*O*-tetradecanoylphorbol 13-acetate. Ribozyme RT-PCR amplification products were seen from ACH2 cell supernatants transfected with crHIV-1.11 DNA (lane 4). No such products were observed from cell supernatants transfected with pGEM 3Z control plasmid (lane 2). Lanes 1 and 3 are RT-PCR negative controls. (*B*) crHIV-1.11 ribozyme DNA production in Jurkat cells infected with supernatants from ACH2 cells previously transfected with crHIV-1.11 DNA (Fig. 5*a*). Amplification of crHIV-1.11 ribozyme DNA was seen (lane 2) only from Jurkat cells infected with cell-free supernatants from crHIV-1.11 cells, while cells infected with mock transfected ACH2 cell supernatants showed no evidence of ribozyme DNA (lane 1). (*C*) Production of virions containing crHIV-1.11 RNA from Jurkat cells transfected with crHIV-1.11 DNA and then superinfected with wt-HIV. crHIV-1.11 RT-PCR amplification products were seen from Jurkat cells first transfected with crHIV-1.11 DNA and then infected with NL4-3 HIV (lane 4). Jurkat cells mock transfected with pGEM-3Z and then infected with wt-HIV did not produce ribozyme amplification products characteristic of crHIV RNA containing virions (lane 2). Lanes 1 and 3 are RT-PCR negative controls. The source of the HIV helper-virus is shown. In panels A and B it is derived from the endogenous HIV in ACH2 cells. In panel C, the HIV helper-virus is NL4-3 HIV that superinfects crHIV containing Jurkat cells.

contained crHIV-1.111 RNAs, whereas both wt-HIV and crHIV-1.111 RNAs were detected intracellularly. Moreover, these cotransfected cells showed a significant decrease in the amount of genomic wt-HIV RNAs. These observations support our hypothesis that ribozyme scission of wt-HIV RNAs occurs most efficiently when genomic wt-HIV RNAs colocalize with crHIV RNAs, as is the case during HIV RNA packaging/dimerization. Scission of wt-HIV RNAs at this stage probably results in the destruction of cleaved genomic wt-HIV RNAs by cellular nucleases and leads to the selective packaging of crHIV RNAs into virions. Our studies found that crHIV-1.111 vectors could interfere with wt-HIV replication for 22 days after cotransfection of Jurkat cells. It should be noted, however, that prolonged durations of wt-HIV interference may not be necessary for the application of crHIVs since infected CD4+ T cells have a relatively short half-life *in vivo* (11, 43–45).

We observed escape virus growth from cultures that were cotransfected with wt-HIV and crHIV DNAs that contained either single or double ribozymes. The escape virus growth seen in our studies is probably due to a functional limitation of ribozymes within cells that allows wt-HIV RNAs to escape intracellular ribozyme cleavage. The intracellular functional limitation of ribozymes is unlikely to be simply attributable to ribozyme concentration because the experiments in Jurkat cells were performed in a molar excess of crHIV DNA (wt-HIV/crHIV ratio of 1:3). This interpretation of the data is supported by our previous findings with HIV genomes containing cis

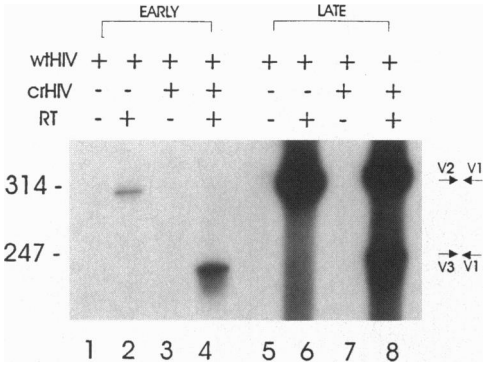

FIG. 6. Analysis of escape virion RNAs. RT-PCR of virion associated RNAs during the early (lanes 1–4) and late (lanes 5–8) stages of virus growth using virus specific primers (V1, V2 and V3). Virions from wt-HIV and crHIV-1.11 cotransfected cultures at the early stage of virus growth (Fig. 2*a*; wt-HIV day +11, crHIV day +19) contained predominantly crHIV RNA and only a small amount of wt-HIV RNA (lane 4). In contrast, virions from wt-HIV and crHIV-1.11 cotransfected cultures at the late stage of virus growth (Fig. 2*a*; wt-HIV day +17, crHIV day +23) contained predominantly wt-HIV RNAs (lane 8). Virions from control wt-HIV and mock cotransfected cultures contained only wt-HIV RNAs during either early or late stages of virus growth (lanes 2 and 6). RT-PCR negative controls demonstrated that the products resulted from amplification of viral RNA (lanes 1, 3, 5, and 7).

anti-U5 ribozymes, where both the ribozyme and target sequences reside on the same strand of RNA (35, 37). Transfection of these cis ribozyme-containing HIV genomes into CD4 cells results in similar escape virus growth results, demonstrating that escape virus growth occurs at sequestering concentrations of ribozyme. It is possible that a certain proportion of intracellularly expressed ribozymes are transcribed in a nonfunctional, perhaps misfolded, conformation (46). Transcripts containing multiple ribozymes may relieve this functional limitation by providing a greater probability for functional ribozymes to hybridize and cleave target RNAs. It is important to note that our conclusions on the nature of escape virus growth only address virus produced from the initial rounds of replication. Our data do not exclude the possibility that mutated wt-HIV or crHIV RNA sequences could form in subsequent rounds of viral replication. Therefore, it may be necessary to construct crHIV vectors that contain multiple ribozymes to different wt-HIV RNA sites for their effective *in vivo* application (47).

DI crHIV vectors are not engineered to intracellularly immunize cells from HIV infection, but to decrease pathogenic wt-HIV loads by competition with wt-HIV. We found that crHIV particles were produced from HIV-infected cells and that crHIV RNA-containing virions were able to infect uninfected cells. Furthermore, crHIV RNAs could be rescued into virions by superinfection of crHIV-containing cells with wt-HIV. Superinfection of crHIV containing cells with wt-HIV occurs because crHIV genomes do not encode for HIV proteins that block superinfection (18–20).

The effective competition of pathogenic wt-HIVs by crHIV vectors may not only require selective packaging of crHIV genomes into virions, but also efficient spread of crHIV genomes to uninfected CD4 cells (11, 44, 45). In this study, we have demonstrated that a selective advantage can be conferred to crHIV genomes by their preferential packaging into virions. We have not addressed the second issue of efficient spread of crHIV genomes to uninfected CD4 cells, which may be important for their effective competition with wt-HIV. It may be possible to design crHIV vectors that effectively compete with wt-HIVs. Such vectors could be therapeutically used to limit wt-HIV loads *in vivo* and decrease their pathogenicity (48–50).

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1. Huang, A. S. & Baltimore, D. (1970) *J. Mol. Biol.* **47,** 275–291.
2. Sekellick, M. J. & Marcus, P. I. (1980) *Virology* **104,** 247–252.
3. Holland, J. J. (1987) in *The Rhabdoviruses*, ed. Wagner, R. R. (Plenum, New York), pp. 297–360.
4. Barrett, A. D. & Dimmock, N. J. (1986) *Curr. Top. Microbiol. Immunol.* **128,** 55–84.
5. Schlessinger, S. (1988) *RNA Genet.* **2,** 167–185.
6. Huang, A. S. (1988) *RNA Genet.* **3,** 195–208.
7. Roux, L., Simon, A. E. & Holland, J. J. (1991) *Adv. Virol. Res.* **40,** 181–211.
8. Saag, M. S., Hahn, B. H., Gibbons, J., Li, Y., Parks, E. S. & Shaw, G. M. (1988) *Nature (London)* **334,** 440–444.
9. Meyerhans, A., Cheynier, R., Albert, J. Seth, M. Kwok, S. Sinsley, J., Morfeldt-Manson, L., Asjo, B. & Wain-Hobson, S. (1989) *Cell* **58,** 901–910.
10. Coffin, J. M. (1992) *Curr. Top. Microbiol. Immunol.* **176,** 143–164.
11. Coffin, J. M. (1995) *Science* **267,** 483–489.
12. Gorelick, R. J. Nigida, S. M., Bess, J. W., Arthur, L. O., Henderson, L. E. & Rein, A. (1990) *J. Virol.* **64,** 3207–3211.
13. Trono, D. Fienburg, M. B. & Baltimore, D. (1989) *Cell* **59,** 113–120.
14. Green, M., Ishino, M. & Loewenstein, P. M. (1989) *Cell* **58,** 215–223.
15. Malim, M. H., Bohnlein, S., Ahuber, J. & Cullen, B. R. (1989) *Cell* **58,** 205–214.
16. Sarver N. & Rossi, J. J. (1993) *J. NIH Res.* **5,** 63–67.
17. Buchschacher, G. L. & Panganiban, A. T. (1992) *J. Virol.* **66,** 2731–2739.
18. Stevenson, M., Meier, C., Mann, A. M., Chapman, N. & Wasiak, A. (1988) *Cell* **53,** 483–496.
19. Benson, R. E., Samfridson, A., Ottimyer, J. S., Doyle, C. & Cullen, B. R. (1993) *J. Exp. Med.* **177,** 1561–1566.
20. Bour, S., Geleziumas, R. & Wainberg, M. A. (1995) *Microbiol. Rev.* **59,** 63–93.
21. Cech, T. R. (1987) *Science* **236,** 1532–1539.
22. Haseloff, J. & Gerlach, W. L. (1988) *Nature (London)* **334,** 585–591.
23. Symons, R. H. (1992) *Annu. Rev. Biochem.* **61,** 641–671.
24. Dropulic B. & Jeang, K. T. (1994) *Hum. Gene Ther.* **5,** 927–939.
25. Baltimore, D. (1988) *Nature (London)* **325,** 395–396.
26. Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, P. A., Stephens, D. A. & Rossi, J. J. (1990) *Science* **247,** 1222–1225.
27. Malim, M. H., Freimuth, Liu, J., Boyle, T. J., Lyerly, H. K. & Cullen, B. R. (1992) *J. Exp. Med.* **176,** 1197–1201.
28. Marasco, W. A., Haseltine, W. A. & Chen, S.-Y. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7889–7893.
29. Pearson, L., Garcia, J., Wu, F., Modesti, N., Nelson, J. & Gaynor, R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5079–5083.
30. Sullenger, B. A., Gallardo, H. F., Ungers, G. E. & Gilboa, E. (1990) *Cell* **63,** 601–608.
31. Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M. & Felgner, P. L. (1994) *J. Biol. Chem.* **269,** 2550–2561.
32. Ledley, F. D. (1995) *Hum. Gene Ther.* **6,** 1129–1144.
33. Woffendin, C., Yang, Z. Y., Udaykumar, L., Yang, N. S., Sheehy, M. & Nabel, G. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11581–11585.
34. Theirry, A. R., Lunardi-Iskandar, Y., Bryant, J. L., Rabinovich, P., Gallo, R. C. & Mahan, L. C. (1995) *Proc Natl. Acad. Sci. USA* **92,** 9742–9746.
35. Dropulic, B., Lin, N. H., Martin, M. A. & Jeang, K.-T. (1992) *J. Virol.* **66,** 1432–1441.
36. Adachi, A., Gendelman, H. E., Koenig, S., Folks, T., Willey, R., Rabson, A. & Martin, M. A. (1986) *J. Virol.* **59,** 284–291.
37. Dropulic, B. & Jeang, K.-T. (1994) *Antisense Res. Dev.* **4,** 217–221.
38. Ruffner, D. E., Stormo, G. D. & Uhlenbeck, O. C. (1990) *Biochemistry* **29,** 10695–10702.
39. Richardson, J. H., Child, L. A. & Lever, A. M. (1993) *J. Virol.* **67,** 3997–4005.
40. Parolin, C., Dorfman, T., Palu, G., Gottinger, H. & Sodroski, J. (1994) *J. Virol.* **68,** 3888–3895.
41. Myers, G., Korber, B., Wain-Hobson, S., Jeang, K.-T., Henderson, L. E. & Pavlakis, G. N. (1994) *HIV Sequence Database* (Los Alamos Natl. Lab., Los Alamos, NM).
42. Sullenger, B. A. & Cech, T. R. (1993) *Science* **262,** 1566–1569.
43. Ho, D. D., Moudgil, T. & Alam, M. (1989) *N. Engl. J. Med.* **321,** 1621–1625.
44. Wei, X., Gosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H., Saag, M. S. & Shaw., G. M. (1995) *Nature (London)* **373,** 117–122.
45. Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, E., Leonard, J. M. & Markowitz, M. (1995) *Nature (London)* **373,** 123–126.
46. Herschlag. D. (1995) *J. Biol. Chem.* **270,** 20871–20874.
47. Chen, C. J., Banerjea, A. C., Harrison, G. G., Haglund, K. & Schubert, M. (1992) *Nucleic Acids Res.* **20,** 4581–4589.
48. Piatak, M., Jr., Saag, M. S., Yang, L. C., Clark, S. J., Kappes, J. C., Luk, K., Hahn, B. H., Shaw, G. M. & Lifson, J. D. (1993) *Science* **259,** 1749–1754.
49. Coombs, R. W., Collier, A. C., Allain, J. P., Nikora, B., Leuther, M., Gjerset, G. F. & Corey, L. (1989) *N. Engl. J. Med.* **321,** 1626–1631.
50. Saksela, K., Stevens, C., Rubinstein, P. & Baltimore, D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1104–1108.