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The Remarkable Frequency of Human Immunodeficiency Virus Type 1 Genetic Recombination

Adewunmi Onafuwa-Nuga and Alice Telesnitsky*

Department of Microbiology and Immunology, University of Michigan Medical School, 1150 West Medical Center Drive, Room 5641, Ann Arbor, Michigan 48109-5620

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INTRODUCTION

A prominent feature of human immunodeficiency virus type 1 (HIV-1) is the genetic breadth and plasticity of its populations (66). Early molecular epidemiology studies revealed several distinct genetic lineages, now called subtypes, within the main group of HIV-1. The discrete albeit overlapping geographic distribution of these subtypes suggested that much of their differences arose via gradual mutagenesis over time, after the introduction of HIV-1 into humans (210). These subtypes are given alphabetic designations such as subtype A, B, or C and are joined in the pandemic today by a few dozen additional strains that contain interwoven genetic segments derived from multiple earlier-recognized subtypes. Because these recombinants spread among patients, they have been designated circulating recombinant forms (CRFs), and emerging evidence suggests that some historically defined subtypes may themselves be CRFs (3). In addition to CRFs, unique recombinant forms far too numerous to cite have been isolated from individual patients (207).

Both recombination and point mutations contribute to the genetic variation in HIV-1 populations. Base substitutions are introduced principally by error-prone DNA synthesis (263) or by the activities of host antiviral factors such as APOBEC3 family cytidine deaminases (56). These processes introduce roughly 1 substitution per viral genome per generation. Thus,

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Michigan Medical School, 1150 West Medical Center Drive, Room 5641, Ann Arbor, MI 48109-5620. Phone: (734) 936-6466. Fax: (734) 764-3562. E-mail: ateles@umich.edu.

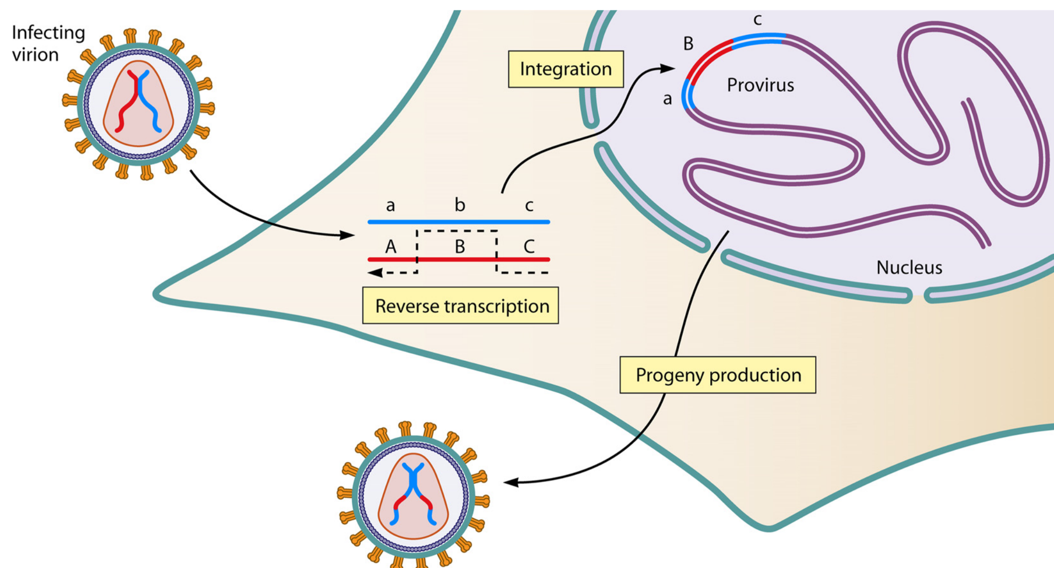

FIG. 1. Pseudodiploid nature of retroviral virions. Virions that package two genetically distinct copies of viral gRNA (red and blue lines) have the potential to generate recombinant proviruses if one or more recombinogenic template switches (indicated with a dashed black line) occur during reverse transcription. Despite copackaging of two gRNAs, retroviruses are not truly diploid because only one allele at each locus is preserved in the integrated provirus, and any progeny produced from a cell harboring a single recombinant provirus will transmit only one allele at each locus in its progeny.

point mutation rates alone are sufficient to explain why retroviruses like HIV-1 exist as snowflake-like quasispecies in which nearly every virus in a population differs from every other one.

Point mutations accumulate fairly steadily over generations and thus can be used to clock viral strain divergence (185). Although selection dictates that variation is not constant across HIV-1 genomes (66), the density of accumulated mutations can be used to determine if a given viral isolate has undergone extensive rounds of replication or has recently been reactivated from a long-established provirus.

In contrast to the clock-like accumulation of genetic change introduced by point mutations, recombination can reset the clock by scrambling genetic content. This can lead to beneficial combinations of mutations, the loss of deleterious mutations, or new starting points for subsequent viral evolution. Whenever clustered substitutions are observed, the variation arose more likely via recombination than by serial point mutations (198, 338). (Hypermutation may be an exception to this rule, although whether such mutations imbedded in less altered genome regions result more frequently from recombination or from the limited processivity of mutagenic factors remains unclear [48, 222, 236].) Some instances of phenotypic switch, including coreceptor switch and reacquisition of drug resistance, have been linked to mutations embedded in localized sequences that differ significantly from flanking sequences, thus providing evidence for recombination within individual patients' virus populations (118, 212, 235, 271).

The propensity of retroviruses to undergo recombination was recognized long before HIV-1 was identified as being the causative agent of AIDS, and thus, evidence for HIV-1 recombination—which was noted as soon as intact viral genomes were sequenced—was not surprising (65). In the 1970s, work with animal retroviruses revealed that markers reassorted so readily that they appeared unlinked (334, 349). Due to the fact that the retroviral genome is a single RNA and, thus, genes cannot physically reassort, this suggested that retroviruses had evolved to recombine their physically linked genes at an unprecedentedly high rate.

Early experiments addressing whether or not HIV-1 could recombine confirmed that recombination was readily detectable. For example, one mutant's stop codon was rescued by recombination with a different defective HIV-1 in tissue culture, and recombination also leads to the cosegregation of drug resistance mutations (60, 163, 220). Because these experiments provided strong selection for recombinants, they could not rule out the possibility that recombination was rare. However, when cultured cells were experimentally coinfected with two distinct strains with similar fitnesses, more than 20% of the proviral population was found to be recombinant, suggesting that recombination was exceptionally frequent (178). Simian immunodeficiency virus (SIV) recombination was readily detected in experimentally coinfected monkeys, demonstrating that recombination of HIV-like lentiviruses also occurs in vivo (100, 347).

HIV-1 recombination does not involve nucleic acid breakage and rejoining but instead results from reverse transcriptase (RT) template switching between viral RNAs during provirus synthesis. Two fundamental properties of retroviruses are critical to their high frequency of recombination. The first is that retroviral genomic RNAs (gRNAs) are encapsidated in pairs. Upon infection of a new cell, the proximity of the two gRNAs facilitates template switching that is orders of magnitude more frequent than that for other viruses. Despite harboring two complete gRNAs per particle, retroviruses are not truly diploid and are best described as being "pseudodiploid" (Fig. 1). This is because only one or fewer DNAs is synthesized per virion, and thus, only one allele at each locus is passed on in the progeny DNA. Part of the reason that no more than one DNA

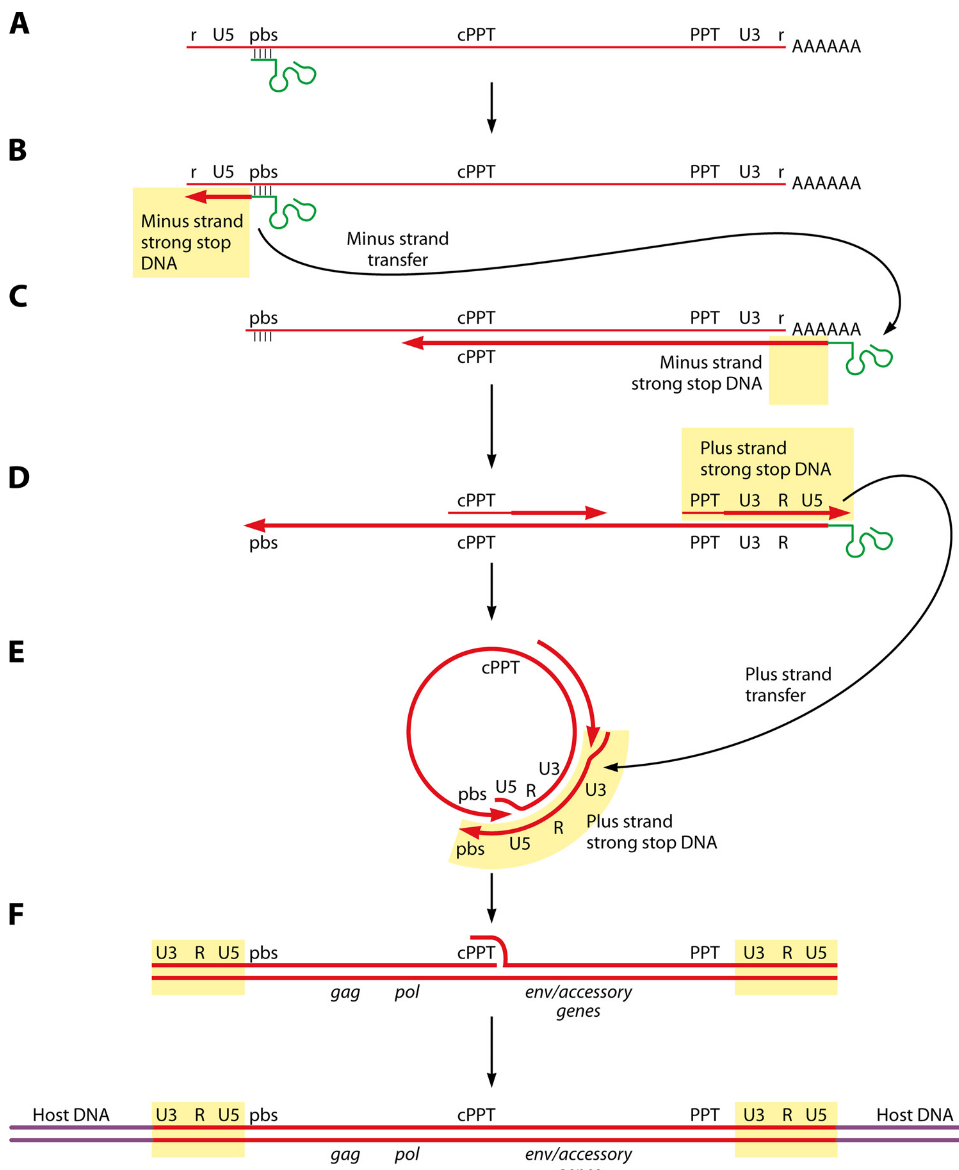

FIG. 2. Process of HIV-1 reverse transcription. (A) A tRNA primer (tRNAlys) is bound to complementary sequences at the primer binding site (pbs) on encapsidated HIV-1 gRNAs. (B) Minus-strand DNA synthesis initiates from primer tRNA and halts when it reaches the 5′ end of gRNA, generating "minus-strand strong-stop DNA." (C) After unmasking of the nascent minus-strand strong-stop DNA by RNase H degradation of the template RNA strand, the replicative template switch known as minus-strand transfer occurs for complementary R-region sequences near the 3′ end of the gRNA, and minus-strand DNA synthesis continues. (D) As minus-strand synthesis proceeds, plus-strand DNA synthesis is initiated from oligoribonucleotides that persist at the PPTs. The nascent plus-strand DNA that results when synthesis is halted at the first modified base in primer tRNA is called plus-strand strong-stop DNA. (E) After the RNase H-mediated removal of the tRNA primer, plus-strand strong-stop strand transfer results from the annealing of the 3′ end of plus-strand strong-stop DNA to complementary sequences at the end of the minus-strand DNA intermediate. (F) DNA synthesis is completed, generating double-stranded DNA with long terminal repeats and a central flap at the central PPT (cPPT). Integrase catalyzes the establishment of the provirus, and host repair enzymes remove flaps and gaps. Thin red lines represent viral RNA, and thicker lines represent viral DNA. Primer tRNA is depicted as the green objects. (Data are not to scale.)

is made per viral particle is stochastic: probably less than 1% of all virions generate infectious proviruses, and thus, the probability of generating two is <0.01%. Furthermore, template switching during minus-strand synthesis all but precludes the generation of more than one DNA per virion due to RNase H degradation of template segments. In this same manner, the high frequency of HIV-1 recombinogenic template switching effectively limits the number of DNAs generated per gRNA dimer to 1 (368).

The second property of retroviruses that is critical to their unusually high recombination frequency is their recombination-prone replication machinery. It was hypothesized that retroviruses are prone to recombinogenic template switching because of the need to perform two mechanistically similar

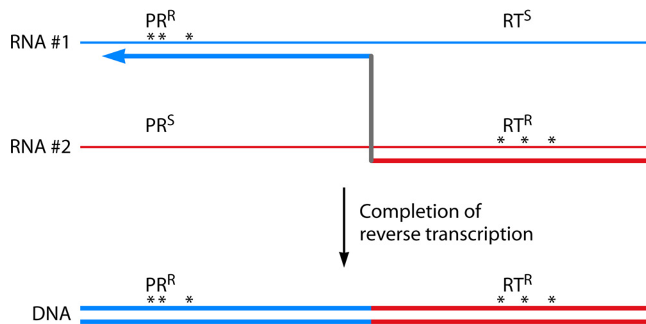

FIG. 3. Recombinogenic template switching. The thin lines (blue and red) represent genetically distinct copackaged gRNAs; thick lines represent viral DNA. The arrow shows the direction of DNA synthesis, and asterisks depict the sites of mutations that confer resistance to either RT or PR inhibitors. Here, template switching generates a recombinant provirus that is resistant to both types of inhibitors.

replicative template switches during every round of viral DNA synthesis (68, 320) (Fig. 2). Retroviral genomes are composed of single-stranded RNAs, designated "plus-strand" (or "sense-strand") RNAs because they contain open reading frames that are recognizable by host ribosomes. The first DNA intermediates synthesized are thus minus stranded, or antisense. The generation of a retroviral DNA is not so simple as the copying of plus-sense RNA into minus-strand DNA, followed by the synthesis of a plus-strand complement. Instead, two replicative template switches, also known as strong-stop strand transfers or "jumps," join and duplicate sequences found only once in gRNA to reconstitute the long terminal repeats at the boundaries of preintegrative DNA (110, 318).

In contrast to strong-stop switches, which occur almost exclusively at defined positions, recombinogenic template switching may occur from any position in the retroviral genome (11). In its simplest form, retroviral recombination involves copying part of one gRNA, followed by RT switching to a homologous region on a copackaged gRNA to complete viral DNA synthesis (64). This can lead to recombinant genomes if the copackaged RNAs contain allelic differences. In the example shown in Fig. 3, one parental gRNA contains a protease (PR) allele with mutations that confer broad resistance to protease inhibitors, while the second genome contains RT sequences that confer zidovudine (AZT) resistance. Because both high-level resistance to AZT and cross-resistance to protease inhibitors require multiple alterations, developing either one can take many virus generations and extensive mutagenic exploration of the fitness landscape (42, 107). In contrast, once each resistance allele has developed independently, recombination permits the cosegregation of both traits in a single cycle of replication.

In this review, the term "genetic recombination" is used to describe the reassortment of viral genome regions. However, the integration of a provirus is arguably the ultimate form of retrovirus-mediated genetic recombination. Also, as discussed below, HIV-1 integration provides a means of persistence and access to host genetic material that can influence recombination outcomes. Although very infrequent, host-mediated recombination among integrated retroviruses and related elements can also occur and can contribute to host evolution (18, 122, 140).

FREQUENCY AND MECHANISM OF HIV-1 GENETIC RECOMBINATION

The current consensus from experimental studies is that HIV-1 recombinogenic template switching occurs at the remarkable rate of roughly every 2 kb or possibly even more frequently under some physiological conditions (11, 50, 275). This section introduces the approaches that led to these conclusions and the properties of virus replication that are responsible.

Experimentally Assessed Recombination Frequencies

Early HIV-1 recombination studies involved infectious virus in tissue culture or, in the case of SIVs, infected animals (178). When examined at early time points, recombination frequencies can be estimated using infectious virus (302). However, it is challenging to synchronize cycles of viral replication due to the lengthy (>3 h) and variable duration of reverse transcription (237). Even modest differences allow more-fit viruses to dominate populations, and therefore, the prevalence of recombinants can be more representative of fitness than of recombination frequency (67). Thus, an accurate assessment of recombination rates requires limiting replication to single rounds (354).

Initial single-round HIV-1 recombination values were based on crossover frequencies in HIV-1 sequences using virus mutants that contained selectable marker genes in place of *env* (148, 355). The HIV-1 strains used, HXB2 and BCSG3, diverge by about 5%, which allowed crossover-site mapping by determining the parentage of genome segments using heteroduplex tracking (148, 355). The results of a labor-intensive analysis suggested that two to three recombinogenic crossovers occur during the synthesis of every HIV-1 DNA. Other researchers subsequently obtained similar or higher frequencies by using either HIV-1 genome-derived or heterologous recombination targets. These results suggest that recombination target sequences do not affect HIV-1 recombination frequencies appreciably (89, 240, 275).

Thus, most subsequent work on HIV-1 recombination frequencies has been performed using reporter assays like those which Howard Temin and colleagues pioneered for gammaretroviruses (135, 136). These assays employ vector and helper systems like those used for retrovirus-mediated gene transfer (Fig. 4). Typically, recombination is monitored by the generation of a functional reporter gene using pairs of vectors, each of which contains a partial or disrupted reporter. Virions harvested from vector-producing cells are applied to target cells in which vector RNAs are reverse transcribed and integrated. When vectors are engineered to contain markers that can be selected by drug treatment or the like, even rare reverse transcription events can be detected by the reconstitution of marker genes (135, 136, 189, 195). Most recently developed systems are scored by means such as flow cytometry, which allows high-throughput analysis.

Repeat deletion has been used as an alternate approach and is based on observations that repeated sequences are often

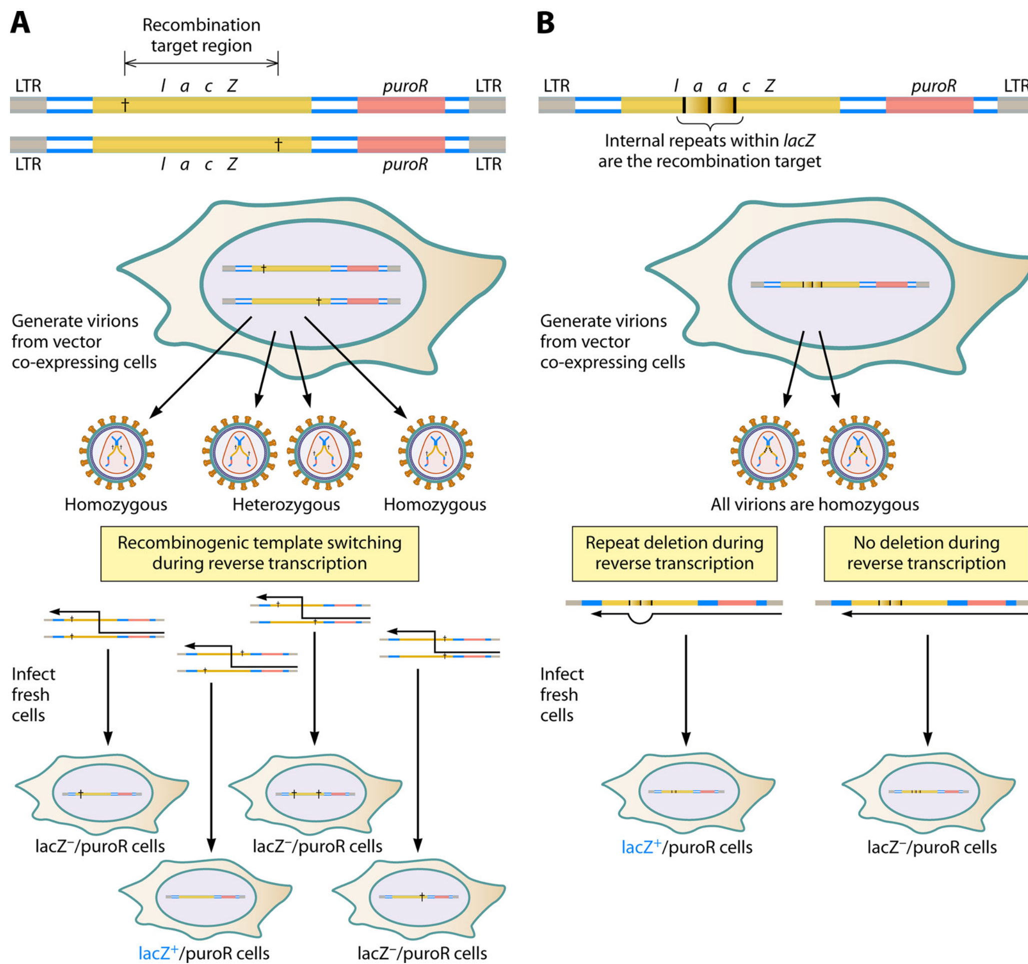

FIG. 4. Retroviral recombination assays. (A) Two vector assay to assess interstrand template switching rates. Each vector has a defect in LacZ-coding sequences, as indicated with the daggers. Each virion generated from producer cells will contain either a homodimer or a heterodimer of gRNAs. Upon infection of fresh cells, recombination between gRNAs within the recombination target region can create a functional *lacZ* gene for virions containing heterodimeric gRNAs. (B) Single-vector assay demonstrating intrastrand recombination by repeat deletion. The vector contains a repeated sequence within the LacZ coding region. In this instance, virions generated from the producer cell all contain gRNA homodimers. Upon infection, functional *lacZ* results from a precise deletion of one of the repeats via an intrastrand template switch. Note that repeat deletion can occur either between repeats on one gRNA or from one gRNA to the other: reciprocal repeat assays have shown that intrastrand and interstrand crossovers occur at similar frequencies (240). LTR, long terminal repeat.

precisely deleted from retroviral vectors (274). Repeat deletion vectors are designed so that deletion inactivates or reconstitutes a marker gene (155, 257, 358) (Fig. 4B). Comparisons of HIV-1 switching between two gRNAs to direct repeat deletion rates suggest that repeat deletion reproduces homologous recombination (240). Because each vector contains both the sequence from which RT will switch (the donor template region) and the sequence to which RT will switch (the acceptor region), repeat deletion assays require only single-vector gRNAs.

Although experimental work defines the average recombination frequency, note that whether or not a crossover occurs at a given template position appears to be largely stochastic, and the spectrum of recombinants within populations is undoubtedly broad. Experimentally, some recombinants displayed at least 10 crossovers, when the average was less than 3 (148). Perhaps the best current estimate of HIV-1 recombination rates is about four to five crossovers per genome, a truly astounding rate that suggests that recombination is a normal part of the generation of each viral DNA.

An obvious limitation of these assays is that they are used to describe HIV-1 replication in culture. Single-cycle experiments are intended to be free of selection, whereas small differences in selective advantage can have profound effects on natural populations (67). Thus, experimental rates of recombination can be compared to clinical outcomes to help assess selective forces. For example, an analysis of intrapatient recombination between the R5 and X4 *env* genes revealed evidence of a coreceptor switch resulting from two recombinogenic template switches occurring within 240 bases in *env* (212), much closer than predicted by average crossover rates but likely detected because of phenotypic selection. The appearance of such variants is likely possible because the very high frequency of crossing over per replication cycle, paired with the estimated $\sim 10^{10}$ virions produced per day, provides HIV-1 with an almost infinite combinatorial potential (252).

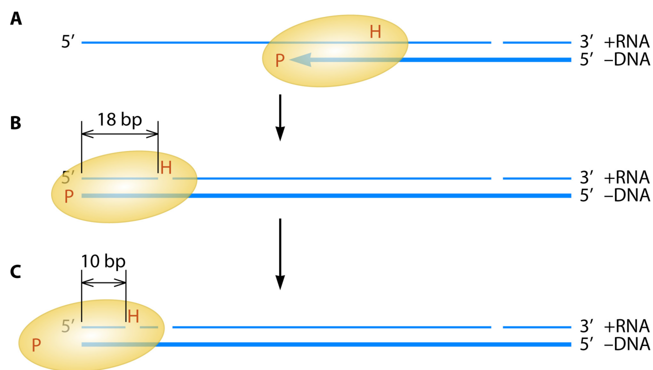

FIG. 5. Interactions of HIV-1 RT with primer-templates. HIV-1 RT is represented by the gold oval, where P indicates the DNA polymerase active site and H is the RNase H active site. The thinner line represents the viral RNA template strand, while the thicker line represents the nascent DNA (primer) strand. The blue arrow shows the direction of DNA synthesis. (A) The DNA polymerase active site is engaged at the primer strand 3′ terminus, and the RNase H active site engages the template strand and performs limited template cleavage as DNA synthesis proceeds. (B) When RT reaches the 5′ end of the RNA template, RNase H cleavage leaves an 18-base oligoribonucleotide annealed to the nascent DNA. (C) Upon RT translocation, which displaces the primer terminus from the DNA polymerase active site, or RT rebinding, further RNase H cleavage generates an 8- to 10-base RNA remnant.

Enzymology of Recombination

Template switching can be recapitulated in cell-free reactions by using model primer-templates and purified RT. The addition of the viral nucleic acid chaperone protein, NC (nucleocapsid), promotes template switching. Reported recombination frequencies in vitro are lower than those observed during viral replication (225, 228). This is likely due in part to physical properties of the reverse transcription complex that results upon virus uncoating. Because some host restriction factors affect early replication steps such as reverse transcription, they may modulate recombination. Indeed, some impairment of RT elongation and replicative template switching has been linked to APOBEC3G (A3G) (31, 144, 205). However, although contributions by host factors cannot be ruled out and host activation of the reverse transcription complex may be required, no factors other than RT and NC are known to contribute to HIV-1 recombination (111, 153, 341).

RT. HIV-1 RT is an asymmetric heterodimer composed of p66 and p51 subunits. The larger p66 subunit contains active sites for both the DNA polymerase of RT and its RNase H activities. The smaller subunit of RT, p51, shares the N-terminal sequence of p66, but p51 differs in tertiary structure and serves principally as a structural subunit in the p66/p51 heterodimer. The distance between DNA polymerase and RNase H active sites corresponds to 17 or 18 RNA/DNA hybrid base pairs (145). Several crystal structures that present RT in association with ligands or poised for catalysis have been solved (288). For the purposes of the discussion here, RT is represented highly schematically as an oval, with "P" indicating the DNA polymerase active site and "H" indicating the active site for RNase H (Fig. 5).

Both DNA polymerase and RNase H activities are required for replication in general and also for recombination (40, 46, 106, 196, 290, 325). The two catalytic sites of RT can engage primer-templates and act simultaneously during elongation. As a result, when RT reaches the 5′ end of an RNA, RNase H cleavage initially leaves a residual ~18-base oligoribonucleotide annealed to the nascent DNA strand (98, 101). Secondary cleavage products 8 to 10 bases in length appear upon RT translocation or rebinding (112) (Fig. 5).

Although limited replication is possible when DNA polymerase and RNase H are provided on separate molecules (154, 319), the coupling of RNase H with DNA synthesis appears to be important for some RT properties, including recombination. However, the joint action of more than one RT may contribute. Retroviruses contain roughly 100 RT molecules per virion (335). Most virions probably contain only two gRNAs, and thus, RT exists in significant molar excess (68). Although the DNA polymerase of RT is so sluggish that DNA synthesis takes several hours, RNase H activity is even slower. Thus, template cleavage is likely aided by RT molecules in addition to those engaged in elongation (78, 159).

Specific interactions between RT and its primer-templates—both the donor template, with which RT associates prior to template switching, and the acceptor template, to which it transfers—affect template switching, since RT mutations that affect primer-template interactions alter recombination, at least for gammaretroviruses (255, 310). Some drug-resistant HIV-1 RTs display increased levels of template switching when assayed in tissue culture (234). For some connection domain mutations that augment AZT resistance, template switching rates decrease (232). For both HIV-1 and gammaretroviruses, reducing RNase H activity decreased template switching (37, 234). These findings suggested that retroviruses with low RNase H activity, such as HIV-2 (132), would recombine less frequently than HIV-1, which possesses robust RNase H. However, subsequent studies demonstrated that rates of template switching are very similar for HIV-2 and HIV-1 (50, 221).

A structural understanding of how RT recognizes and recruits acceptor templates, releases donor templates, and completes recombination will require additional research; however, recent work may be bringing us closer by suggesting that elongating HIV-1 RT is much more of a contortionist than previously recognized (2). Single-molecule fluorescence resonance energy transfer studies of substrates representing the polypurine tract (PPT) have shown that RT can rapidly flip between alternate binding orientations for plus-strand primer generation and primer utilization (2). Follow-up work revealed that RT is a "restless" enzyme, rapidly sliding between template ends until it locates the primer 3′ end. Its dwell time there is modulated by how firmly the fingers and thumb domains clamp in place (192). One remarkable feature of these binding-mode dynamics is that they occur without RT dissociation. The possibility that approaches like these may be adapted to address questions such as whether or not template switching requires complex dissociation or if template switching occurs from a discrete position in the chemical cycle of RT is an exciting possibility (159, 277).

NC. HIV-1 NC affects reverse transcription outcomes in vitro, and the defects of some NC mutants confirm likely roles in DNA synthesis. NC is a small, highly basic protein that binds RNA and functions as a nucleic acid chaperone (188, 273). As a domain of the Gag precursor, NC specifies gRNA recruit-

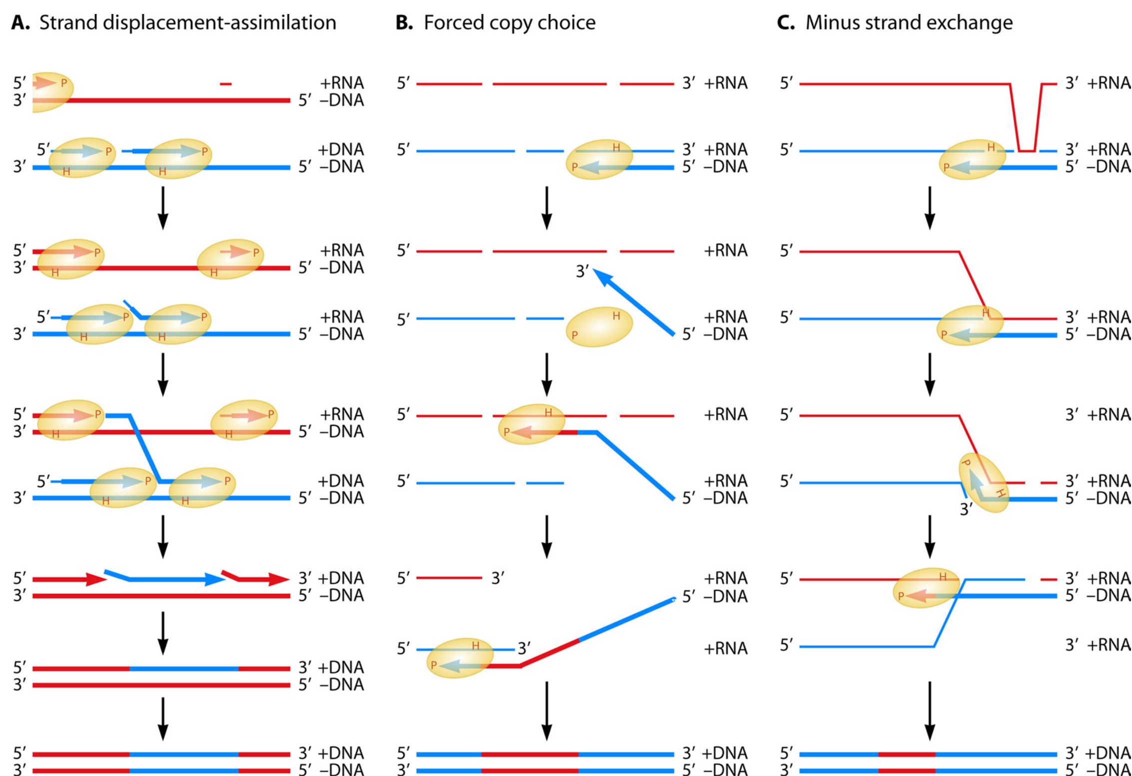

FIG. 6. Three models for retroviral recombination. (A) Strand displacement-assimilation showing recombination during plus-strand synthesis. (see the text and reference 160). (B) Forced copy choice (see the text and reference 68). (C) Minus-strand exchange (see the text and reference 26). Thin lines are retroviral RNAs with breaks in the strands. Thick lines represent the nascent DNA; arrows indicate the direction of DNA synthesis. Gold ovals represent HIV-1 RT.

ment via high-affinity protein-RNA interactions. As a processed peptide, NC coats gRNA at a stoichiometry of about 1 NC per 7 to 8 nucleotides (352). Although NC binds single-stranded RNA preferentially and induces nucleic acid melting, it also promotes annealing and strand exchange (188, 328, 353). NC has been implicated as a part of the recombination machinery and affects template switching in reconstituted reactions (226, 227, 280). Other work suggested that NC may aid acceptor template recruitment and inhibit nonhomologous recombination (128).

Many NC recombination effects likely mirror its general roles in reverse transcription. NC promotes RT elongation and reduces self-priming and pausing at template structures (119, 183). NC may aid the annealing of invading acceptor templates and the unmasking of primer strand DNA, and one study showed that NC brought the site of transfer closer to the apparent invasion site (124). In a study of murine leukemia virus (MLV) reverse transcription on structured templates that increased switching fivefold, NC zinc finger mutants increased the recombination rate a further sixfold (364). Some NC mutants that affect the kinetics of HIV-1 reverse transcription have also been reported (322).

Models for Retroviral Genetic Recombination

Extensive literature suggests that HIV-1 genetic recombination results from a copy choice mechanism, that is, the alternating use of two templates during the synthesis of a single viral DNA (11). In concept, retroviral recombination could result from template switching during either minus- or plus-strand DNA synthesis, and distinct models have been proposed for each one (64, 160, 225).

The "strand displacement-assimilation" model describes recombination during plus-strand synthesis and was based on electron micrographs of partially reverse-transcribed avian retrovirus genomes visualized after virion disruption (157, 158). The images revealed DNAs joined in "H"-branched structures suggestive of the recombination intermediates shown in Fig. 6A. These structures were postulated to result after plus-strand synthesis initiated from multiple positions and strand displacement yielded free DNA tails that could invade neighboring strands. Some evidence of alternate plus-strand starts was reported for HIV-1 (172, 321), as was plus-strand recombination in reconstituted reactions (99). However, most HIV-1 plus-strand synthesis initiates at only two positions: the 3′-terminal and central PPTs. Strand displacement synthesis terminates shortly after it initiates at the central PPT, generating a DNA flap that has been implicated in several replication processes but likely does not promote appreciable recombination (88). Neither major nor reported minor plus-strand starts coincide with reported crossover hot spots (21, 172), and no experimental evidence suggests that HIV-1 plus-strand recombination occurs frequently.

The roles of the RNase H activity of RT provide additional support for the likelihood that most recombination occurs dur-

ing minus-strand synthesis (40, 46). Other evidence comes from observations that recombination is fully as frequent when only minus-strand recombination is possible as when both minus- and plus-strand recombination could contribute (12, 148, 355, 359). Another argument against significant plus-strand recombination lies in the very high frequency of minus-strand crossovers, because the template degradation which that would require precludes extensive plus-strand diploidy (221). Nonetheless, "backwards" insertions have been observed for both experimental replication products and clinical isolates (87, 311; see discussion in reference 314). This demonstrates that minus-strand DNAs occasionally serve as recombination templates and leaves open the possibility of some plus-strand recombination.

The "forced-copy-choice" model explains how template switching could generate an intact proviral DNA from fragmented gRNAs and was based on observations that gRNAs isolated from animal retroviruses often appeared nicked and yet efficiently generated infectious viral DNA (68) (Fig. 6B). In this model, nascent DNAs dissociate from donor gRNA templates when nicked template ends are reached, allowing primer strands to associate with regions of acceptor homology on the copackaged gRNA. A model variation based on the high frequency of repeat deletion suggests that RNA breakage is not required (350).

A second minus-strand recombination model, called "minus-strand exchange" or "acceptor invasion," is currently the favored model for most recombination (37, 64, 251, 281) (Fig. 6C). RNase H-mediated template degradation exposes nascent minus-strand DNA, allowing base pairs between DNA synthesized on one gRNA and complementary regions on the second gRNA. Primer strand realignment by branch migration follows, with the resumption of DNA synthesis on the acceptor template yielding the recombination junction. Data from studies in vitro using purified enzymes and model primer-templates provide support for key predictions of this model (53, 104, 125). These predictions include reports of a preswitch complex that includes both donor and acceptor templates (251), the stimulatory effects of RNase H activity (37, 142), and effects of occluding acceptor invasion docking sites (125). In the mode of acceptor recruitment, via base pairing between the primer and acceptor strands "behind" the crossover site, "acceptor invasion" resembles the strand displacement-assimilation model, whereas in the replication stage targeted, it resembles "forced copy choice."

Examinations of recombination rates and complementation frequencies of RNase H mutant RTs formed the basis of a model variation, called "dynamic forced copy choice." This model suggests that a balance between polymerase-dependent and -independent RNase H activities determines recombination rates (142). This model suggests that RNase H activity is necessary for the dissociation of the template-primer prior to template switching (233) and contrasts with minus-strand exchange, which envisions a preswitch complex containing both donor and acceptor, rather than donor template release before acceptor recognition (26, 251).

The acceptor invasion model implies that donor-acceptor homology "behind the growing point" is more important than homology precisely at the crossover site (26). Indeed, RT is capable of significant mismatch extension upon template switching both in vitro and during viral replication (69, 80, 199, 202, 254, 256, 264). At least for MLV, there is a sharp drop-off in the efficiency of acceptor recognition below a threshold homology length that corresponds roughly to the length between RNase H and DNA polymerase active sites and then an additional abrupt drop in recognition for acceptors with only eight or fewer bases of donor template identity (73, 87, 256). Together, these findings suggest that regions of donor-acceptor homology, behind the growing point for DNA synthesis but contained within the RT footprint, are necessary for efficient homologous recombination.

Nonhomologous recombination, which occurs about 100-fold less (361), often reveals junctional microhomology (87, 120, 245, 248, 311, 332, 360). One analysis of over 100 nonhomologous crossovers found junctional microhomology in about 90% of crossovers (87). Thus, in contrast to homologous recombination, which is highly efficient and is dependent principally on donor-acceptor identity behind crossover points, nonhomologous recombination is relatively inefficient and dependent largely on primer terminus-mediated acceptor recognition.

The bottom line is that most retroviral recombination occurs during minus-strand synthesis and relies on base pairing between acceptor template sequences downstream of recombination crossover sites and primer strand sequences unmasked by RNase H activity. A minor form of recombination can guide free primer strand termini to alternate acceptors.

Fidelity of Recombination

The possession of two complete gRNAs sets retroviruses, including HIV-1, apart and raises the question of why these viruses possess two genome copies when one is sufficient for all other viruses (335). The notion that two templates might allow recombination is at least as old as the forced-copy-choice model (68). Because template switching could promote intact genome synthesis from fragmented gRNAs, recombination is predicted to aid faithful genome replication.

Several reports from the 1990s of base substitutions at crossover sites generated in vitro led to the alternate hypothesis that genetic recombination might contribute to HIV-1 mutagenesis (80, 250). If the nontemplated addition of an uncoded base at a template end were followed by mispair extension upon transfer, then most base substitutions in HIV-1 might represent sites of genetic recombination (247, 250).

To address this hypothesis, several studies tested whether or not misincorporation occurred more frequently at recombination junctions than during synthesis on single templates. With the exception of findings for replicative "strong-stop" switches (176), all data determined that substitution rates at crossovers resembled whole-genome rates during virus replication both for gammaretroviruses (256, 362) and for HIV-1 (30, 89, 367). Thus, in contrast to outcomes for cell-free reactions, recombinogenic switching during HIV-1 replication is not particularly error prone.

It was suggested that recombination may aid error escape when RT reaches a dysfunctional environmentally modified base (79). Indeed, a somewhat analogous process is known to occur at the plus-strand transfer step of reverse transcription, when RT reaches the first modified base of primer tRNA

(313). Previously reported observations that DNA synthesis fidelity was drastically reduced for both MLV and HIV-1 when template switching was suppressed by RNase H limitation are also consistent with contributions of recombination to error avoidance (37, 234).

Further support for the notion that recombination aids replication fidelity comes from a study where recombination was prevented by engineering HIV-1 virions to contain only single intact gRNAs (166). The frequency of successful provirus generation was compared to that for normal pseudodiploid genomes. Marker genes were inactivated about threefold more frequently in the absence of the store of genetic information that a second template provided. Genes were inactivated by either point mutations or deletions, many of which were templated by rare spliced gRNAs. More one-gRNA products contained deletions than did two-gRNA ones. When replication was halted before the average half-time of provirus completion, deletion products like those observed for one-gRNA virions were abundant. These findings suggest that HIV-1 replication errors occur less frequently when a second gRNA is present and that full-length proviruses are synthesized more efficiently in the presence of a second gRNA (166).

Figure 7 presents a model for RT elongation that considers these observations in light of the model of minus-strand exchange. Prior to the switch, a duplex composed of nascent minus-strand DNA and the acceptor template exists. This may stabilize the RT elongation complex and aid processive synthesis by facilitating recovery from genome breaks or other blocks to elongation. In the absence of a second gRNA, a broken template or damaged template base would lead to either mutation or aborted synthesis. Findings that the average length of DNA synthesized on single-copy templates appears shorter than average homologous crossover distances are consistent with the possibility that processive RT elongation complexes may include both donor and acceptor templates (87). It is tempting to speculate that some of the cryptic nucleic acid binding sites on exposed surfaces of HIV-1 RT, which complicate efforts to produce uniform preparations of RT poised at the 3′ ends of primer strands, may contribute to these additional binding functions (137, 138).

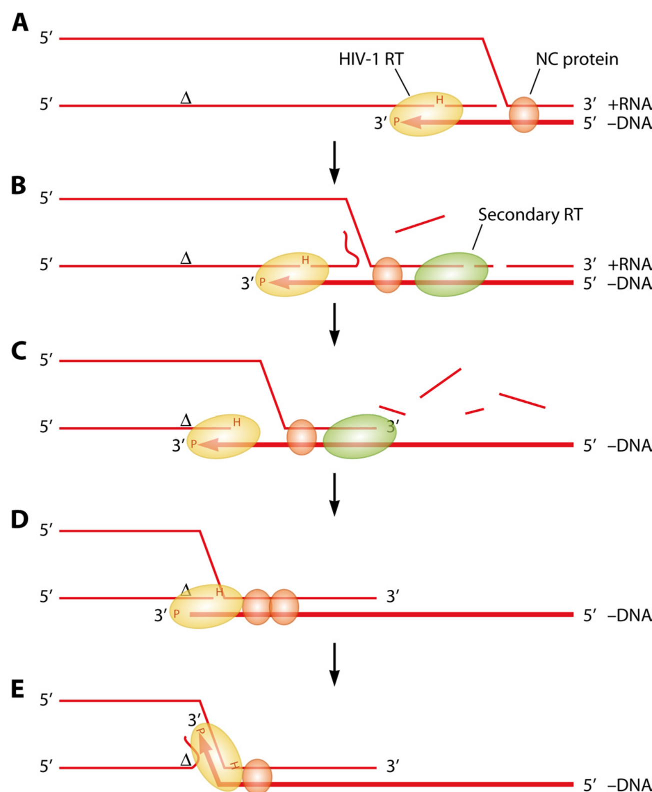

FIG. 7. Speculative model for the processive RT elongation complex. (Based on findings reported in references 44, 79, 166, 251, and 280.) (A) Ordinarily, during elongation by RT, RNase H-mediated template strand nicking provides an opportunity for NC protein-facilitated docking of the acceptor template strand onto the nascent DNA primer strand, behind the elongating RT. (B) As the elongating RT continues DNA synthesis on the donor template, secondary RT molecules further degrade template strands, and branch migration brings the acceptor template in proximity to the elongating RT: this is the putative processive RT elongation complex. (C) When RT reaches an impediment to elongation, RNase H activity "catches up" with the elongating RT. (D) Unable to continue DNA synthesis on the donor template, the polymerase active site disengages, and RT translocates as it does at template ends (Fig. 5C), reducing the length of the RNA-DNA hybrid. (E) Further branch migration displaces the residual oligoribonucleotide and forces primer strand realignment onto the homologous portion of the copackaged gRNA, allowing DNA synthesis to proceed, and the eventual completion of an intact provirus. Gold ovals represent HIV-1 RT molecules that contribute to DNA synthesis; green ovals represent secondary RTs that contribute polymerase-independent RNase H activity; circles represent NC (stoichiometry and locations are highly speculative); triangles represent damaged template positions.

RNA Copackaging Determinants: Producer Cell Coinfection

Genetic recombination can result when a virion containing two distinct gRNAs infects a fresh cell and is not detected when a cell is coinfected with two genetically distinct viruses (Fig. 8) (135, 305). At least two properties are required to generate a virion with two different gRNAs: (i) the establishment of a single cell with two or more genetically distinct proviruses and (ii) the association of two different gRNAs in an encapsidated dimer. The determinants of the RNA dimer partner selectivity differ among retroviruses, and the replication properties of HIV-1 make it particularly prone to generating heterodimeric gRNAs.

Patient coinfection. With the exception of strains that evolve over time, the coinfection of a patient is an obvious prerequisite to cell coinfection. Whether this results more often from the transmission of more than one strain before the establishment of protective immune responses or from subsequent reinfection (superinfection) was not clear initially. However, many clear cases of superinfection and even triple infection have now been described, as has recombination between superinfecting and preexisting patient strains (4, 6, 32, 58, 93, 230, 253, 265, 269, 278, 298, 330). Contemporaneous dual infection is limited by the genetic bottlenecks often observed upon transmission (193). In fact, one recent study involving single-genome sequencing of viruses from over 100 patients concluded that a single infectious particle initiated infection in nearly 80% of the patients (162). Although neutralizing antibody deficiencies may sometimes contribute to susceptibility, patient superinfection can occur even after immune response

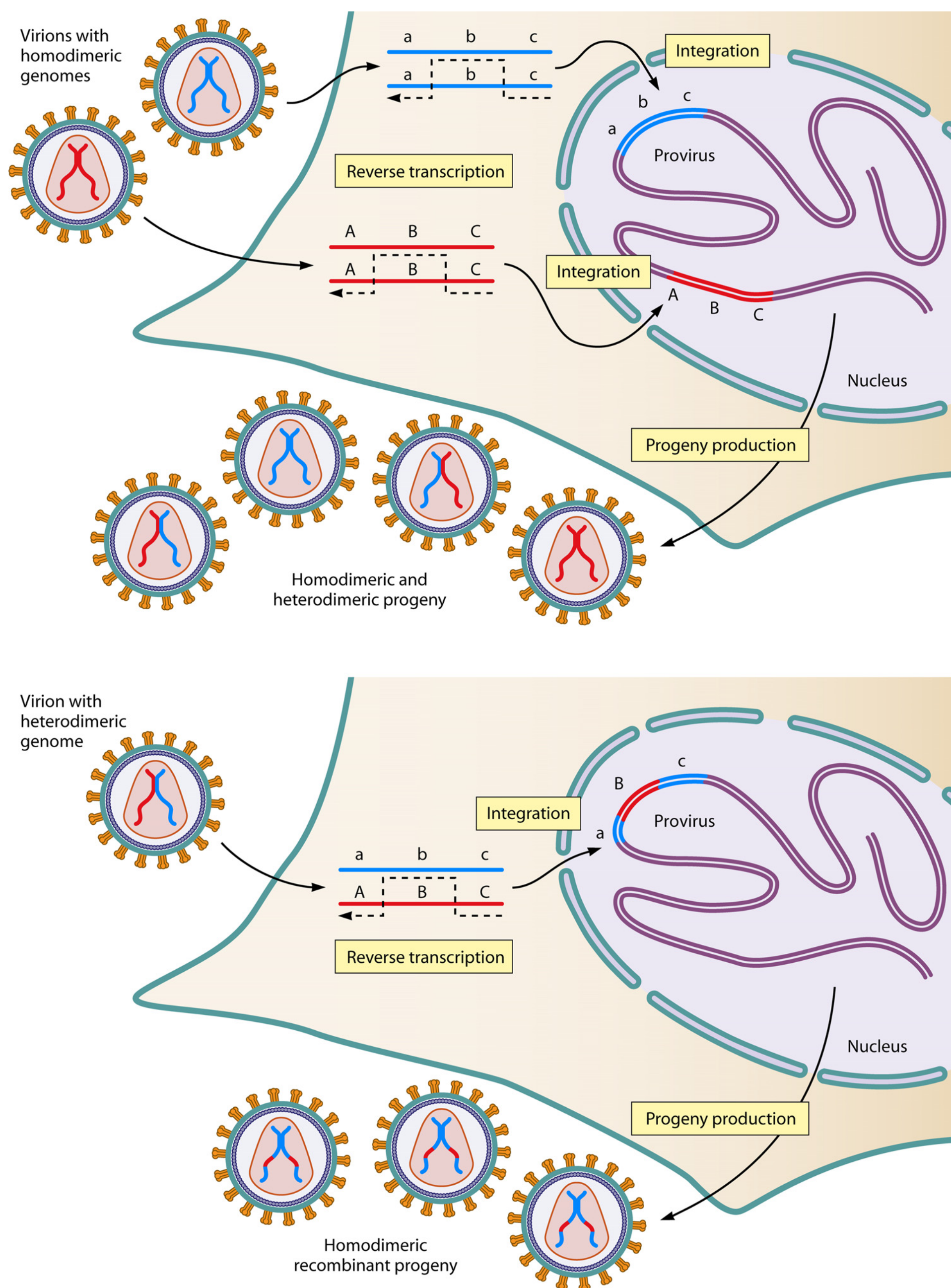

FIG. 8. Requirements for generating recombinant genomes. (A) Coinfection with two genetically distinct viruses does not yield recombinants. However, a producer cell must be coinfected with two genetically distinct viruses (shown here as viral particles with two blue or two red RNAs) to produce viral particles with heterodimeric gRNAs. (B) Recombination is observable in cells infected with heterodimeric virions (particle containing one red and one blue RNA strand). Template switching during reverse transcription can generate a recombinant provirus.

development occurs (259, 299). It was suggested that high-frequency recombination not only may result from coinfection but also may contribute to the establishment of superinfecting strains by allowing the rapid evasion of established immune responses (304). The failure of vaccine trials provides further testament to the challenges of generating immune responses that limit subsequent infection.

Coinfection of an individual does not invariably lead to recombination. For example, although naturally arising recombinants have been reported for HIV-2 (206, 315, 351), as have experimental recombinants between HIV-1 and HIV-2 (217), no natural HIV-1/HIV-2 recombinants have been reported (71, 210) despite high rates of dual infection (327). Reasons for this may include limited cell coinfection and differences in RNA packaging (161, 217, 265). Additionally, the compartmentalization of virus strains to particular organs or other isolated sites can limit effective population sizes and thus the potential for recombination (7, 171, 223).

Cell coinfection. Most retroviral infections display resistance to superinfection or the protection of an infected cell from

subsequent reinfection by a similar virus (230). A common form of superinfection resistance results from a reduction of receptors on the cell surface. This is due to interactions between envelope protein expressed by the resident provirus and the receptor either intracellularly or at the cell surface (127). For simple retroviruses, cell infection by one virus generally limits reinfection by a second virus that shares the same receptor (343).

HIV-1 Nef downmodulates CD4 expression and that of both major coreceptors, and Vpu also contributes to receptor downmodulation (181, 282, 344). The fact that two different accessory proteins contribute to receptor downmodulation suggests that this is important to HIV-1 in vivo. These effects, paired with the short half-life of infected cells, suggest that individual cell coinfection is rare. However, high frequencies of HIV-1 recombination suggest that superinfection resistance does not prevent cell coinfection in vivo.

In situ hybridization of patient-derived cells suggested that it may not be unusual for individual cells to contain four or more proviruses in vivo (114, 156, 265). Although experimental validation of these observations is not plentiful, this is consistent with the very high frequency of genetic recombination in HIV-1 populations. Multiply infected cells may result from the reinfection of cells containing defective or latent proviruses or from transmission properties that result in the simultaneous delivery of multiple virions.

An attractive model with limited experimental support is that dual or higher-order cell infection may sometimes arise via cell-mediated infection (72). In cell culture, when primary dendritic cells or a cell line engineered to express DC-SIGN was cocultured with virus and target cells, increased dual infection was observed (72). Nonrandom coinfection has also been observed for other tissue culture systems (49). Emerging evidence highlights the importance of cell-to-cell spread at neutralization-resistant virological synapses (52, 91, 150). This mode of transmission not only may facilitate virus spread undeterred by host responses but also may enhance the individual-cell coinfection that is a necessary prerequisite to recombination.

Another possibility is that expressed, unintegrated DNA may contribute recombination templates. In experimentally infected $CD4^+$ T cells, unintegrated DNA is in vast excess of integrated proviruses (279). When present on their own, unintegrated HIV-1 DNAs tend to express only spliced RNAs due to low levels of Rev (348). However, a recent report suggested that unintegrated DNAs can complement defective integrated HIV-1 (108). Under these conditions, some unspliced RNAs templated by unintegrated DNAs entered the packageable RNA pool, and recombinants containing genome portions from both integrated and nonintegrated DNAs were observed in subsequent rounds of replication. Although the dynamics of dual infections may be quite different in vivo, unintegrated DNA can be generated under conditions that are not hospitable to provirus generation and appears to be remarkably long-lived in viral latency and in patients whose virus is effectively suppressed by antiretroviral therapy (293). Thus, it is possible that unintegrated DNAs may contribute to recombination in vivo.

Virion RNA Copackaging

The reason why genetic recombination occurs during heterozygous virion infection but not during coinfection is probably local concentration effects. Although anecdotal evidence for recombination-mediated incorporation of target cell sequences was reported for alpharetroviruses (239), and gammaretroviruses occasionally recruit recipient cell primer tRNAs (291), it is likely that even substrates for nonhomologous recombination are generally coencapsidated with gRNAs.

Reverse transcription occurs within the nucleoprotein complex that forms upon virion entry and not free in the cytoplasm (36, 94). In purified reactions, conditions that enhance local concentration, such as joining RNAs in dimers, enhance recombination (13, 23). In contrast, two sequences that are widely separated in primary sequence recombine at least as well as two closely spaced sequences during viral replication, suggesting that copackaging provides sufficient template proximity to promote recombination (76, 240; W. An and A. Telesnitsky, unpublished data). Back-of-the-envelope calculations, based on the length of an RNA A-helix and the diameter of a virion core, suggested that copackaged gRNAs are condensed far more than 30-fold their length (173). Thus, the encapsidated nucleoprotein more likely resembles the inside of a baseball rather than the loosely packed RNA "wishbone" that is usually drawn, and sequences near dimer linkages are likely no closer to their copackaged homologs than to any other sequences.

Because there was no reason to expect otherwise, it was long assumed that gRNAs in dually infected cells would associate at random for packaging (65). If each gRNA were equally as likely to dimerize with one of its siblings as with a genetically distinct gRNA, dimers would form in homodimer-heterodimer-other homodimer proportions predicted by the Hardy-Weinberg equation, or 1:2:1 if the two RNAs were expressed equally (244). Assumptions of random RNA associations were critical to early recombination calculations in which the ratios of the two parental genomes were used to calculate the "recombining population" size or fraction of virions containing heterodimers and thus being capable of generating recombinants (136, 354).

However, this assumption is invalid for some retroviruses. Copackaged gRNAs must possess compatible dimer linkages, and some retroviruses possess additional restrictions that limit which RNAs can serve as dimer partners. Most mechanisms that are known to restrict gRNA partner selection do not act during HIV-1 replication, and this relative promiscuity in RNA copackaging contributes to the recombination rate of HIV-1.

gRNA versus mRNA selectivity. Retroviral gRNAs are capped and polyadenylated like mRNAs, and gRNAs and *gag-pol* mRNAs are indistinguishable in primary sequence. How, where, or whether unspliced RNAs are partitioned into genome and mRNA functions is still unclear and probably differs among retroviruses (25, 41, 70, 312).

What specifies the enrichment of gRNAs over random host mRNAs is a 5′ region called Ψ (psi). HIV-1 RNAs that lack Ψ are packaged preferentially over bulk mRNA (29, 130, 179), which are incorporated largely at random, with a modest enrichment of some mRNAs over others (284). NC, as a domain of the Gag precursor, is the viral determinant of gRNA encapsidation (29).

Some retroviruses do, and others do not, selectively pack-

age each other's gRNAs (45, 161, 303). Although host range restrictions outside the laboratory generally prevent dual infections such as Mason-Pfizer monkey virus plus feline immunodeficiency virus, the possible contributions of packaging restrictions to preventing recombination, and, thus, augmenting vector gene delivery safety, have been described (39, 303).

gRNA dimer versus monomer selectivity. Retroviruses encapsidate gRNAs in pairs even when RNAs are limiting and each particle contains less than one on average (131, 187). Why is this? It is unlikely that one RNA is insufficient due to mass, because particles can form without gRNA. Both MLV and HIV-1 are capable of packaging Ψ-positive (Ψ^+) RNAs more than twice the native length (177, 295). Similarly, host and virus factor alterations can modulate RNA packaging, which demonstrates flexibility in packaged RNA composition (200, 218). Although some retroviruses may copackage more than two RNAs when gRNAs are short (285), and it remains unclear if most of them contain precisely two RNAs, it seems likely that if variation exists, it is in increments of 2—0, 2, 4, or more copackaged gRNAs. This is because the gRNAs in retroviral particles are packaged as non-covalently-linked dimers (272).

Dimerization is likely nucleated by base pairing between palindromic loops, called dimerization initiation sites (DISs), that cap hairpins present on each RNA. This loop-loop "kissing" is followed by dimer interface expansion (85, 186) (Fig. 9). Although some mutations partially separate these functions, overlapping genome segments contribute to both dimerization and packaging (243, 272).

Elegant nuclear magnetic resonance studies with MLV have provided structural insight into "two-or-none" gRNA packaging. Differences between MLV monomeric and dimeric RNA folds unmask high-affinity NC binding sites that promote dimer packaging (85). Although HIV-1 dimer recruitment remains less well understood, several lines of evidence support the likely specific recruitment of at least weak dimer linkages, including findings showing that placing two dimer linkage sequences on a single RNA results in monomer encapsidation (286). However, nascent dimer linkages are relatively labile, and some researchers have suggested that HIV-1 gRNAs are monomeric when first recruited (301).

HIV-1 RNA heterodimerization. Because the packaging of two different gRNAs in a mixed heterodimer requires dimer linkage, recombination should be critically dependent on dimer compatibility. Findings from in vitro reactions support this (115). However, mutations that disrupt RNA dimerization in purified reactions often display less severe defects during virus replication, suggesting that there are multiple ways of initiating gRNA dimerization (301).

Among natural HIV-1 isolates, the palindromic sequences that cap DIS stem-loops vary so that, for example, subtype B genomes should be incapable of dimerizing with subtype A (61, 63). If such differences prevent heterodimerization, and heterodimerization is a prerequisite to copackaging, then recombination between gRNAs that differ in DIS sequences should be much lower than that for gRNAs with the same sequences. Because clinical isolates resulting from recombination between seemingly incompatible strains are known, it is clear that these

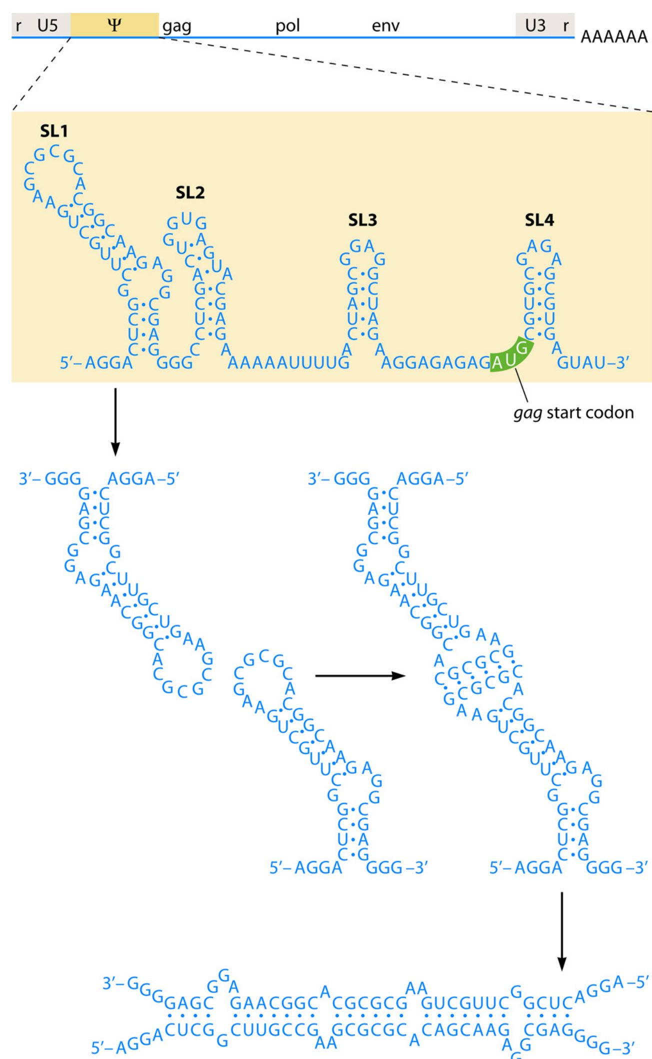

FIG. 9. RNA secondary structure in the packaging and dimerization region of HIV-1 gRNA. A region near the 5′ end of the genome, termed Ψ, is responsible for the selective recruitment of gRNA and is coincident with elements required for dimerization between copackaged RNAs. Here, the area is enlarged to show stem-loop 1 (SL1) to stem-loop 4. The *gag* start codon is indicated as shaded residues; note that alternate models for the 5′ end of HIV-1 gRNA do not include stem-loop 4 but instead evoke long-distance interactions between sequences encompassing the *gag* start codon and U5 regions (1). Stem-loop 1 is capped by a palindromic sequence known as the DIS, which forms a putative kissing-loop interaction that initiates dimerization between two copackaged gRNAs. (Roles and models for these regions are reviewed in references 72, 186, and 216.)

differences do not block recombination entirely. However, clinical observations cannot resolve whether recombinants are detected due only to selective advantage.

St. Louis and colleagues sought to quantify the effects of DIS variation on recombination by using replication-competent virus with DIS loops predicted to be incapable of heterodimerization (302). Diminution of recombination associated with differing DISs, although observable, was much lower than predicted if DIS-mediated heterodimerization were essential (302). In single-replication-round assays, the possession of

compatible DIS sequences was found to enhance heterozygous virion formation. Specifically, when two DIS variants were generated, one with six C residues and the other with six G's, both heterodimerization and recombination increased. Having noncomplementary DIS loops reduced recombination but did not eliminate it: the reduction was less than 1 order of magnitude (54, 55, 215). The conclusion that loop incompatibility does not prevent recombination was confirmed with B/E recombination in an experimentally infected chimpanzee (100).

In summary, compatible dimer linkages enhance heterodimer formation, and strains with incompatible DIS sequences form heterodimers less readily and recombine less often. Such effects may limit recombination in patients as well as in tissue culture (175). However, recombination for strains with mismatched linkages is readily observed and likely reflects a capability to establish dimer linkages even when DIS loops are noncomplementary albeit at reduced levels (301).

Packaging *cis* preference. The ability of certain retroviruses to package RNAs that do not encode viral proteins is essential to retrovirus vectors and represents *trans* packaging. In contrast, some retrotransposons, such as L1 elements, display *cis* preference or the preferential encapsidation of gRNAs from among those that participate in translation (342). *cis* packaging may be advantageous in ensuring that actively replicating elements retain viability within a sea of defective L1 elements.

Some retroviruses also display *cis* preference: notably, HIV-2 preferentially recruits its gRNA from the productive *gag* mRNA pool. The advantage of *cis* preference likely reflects that, unlike HIV-1 and MLV, HIV-2 packaging signals are present on both spliced and unspliced viral RNAs (116). Although HIV-1 and MLV are both capable of packaging Ψ^+ RNAs that have not served as mRNAs (231), differences in how they partition RNAs affect their recombination rates.

gRNA reassortment differences between HIV-1 and gammaretroviruses. Despite the complex mosaic structures observed for natural HIV-1 isolates, the extrapolated high frequency of HIV-1 genetic recombination was surprising initially (355). This was because it was already known that gammaretrovirus recombination was significantly less frequent, with approximately one recombination event during the synthesis of every two to seven gammaretrovirus DNAs (135, 136, 151), and it was assumed that HIV-1 frequencies would be similar. When initial studies suggested that HIV-1 recombination occurred 10-fold more often, species-specific differences in template switching appeared to be the most likely cause (148). However, repeat deletion assays revealed no differences between HIV-1 and MLV (9, 240). This suggested that enzymology was not the primary cause of genetic recombination differences.

An alternate hypothesis for why relatively few gammaretroviruses are recombinant was that only a subset of particles copackaged two different gRNAs (68). If two identical gRNAs became copackaged more frequently than random copackaging would predict, then even if template switching occurred at a uniform and high frequency, marker cosegregation would be reduced.

Experimental support for this alternate hypothesis has now been reported by several groups, who showed that differences in RNA trafficking, and not template switching, cause differences in MLV and HIV-1 genetic recombination (95, 165, 270, 368). Biochemical approaches demonstrated that when two MLV Ψ^+ RNAs were coexpressed, most were encapsidated as RNA homodimers. In contrast, HIV-1 gRNAs formed dimers in random proportions, indicating that commitment to dimerization occurs at different replication stages for these two viruses (96). More random gRNA dimerization is observed when MLV gRNAs are expressed from the same or adjacent transcription units than from spatially separated proviruses, suggesting that gammaretrovirus gRNAs dimerize very early after transcription, before nuclear exit (97, 165). In contrast, HIV-1 gRNAs appear to associate in the cytoplasm. These biases in gRNA selectivity alone appear sufficient to account for observed differences in recombination rates between HIV-1 and gammaretroviruses (95, 368) (Fig. 10).

These findings also explain the phenomenon of "high negative interference," or a highly nonrandom pattern of genetic crossovers, which had long been recognized for gammaretroviruses (15, 68, 133). Most gammaretroviruses show no genetic evidence of recombination. However, among recombinants, most possess more than one crossover, and some display several. "High negative interference" among gammaretroviruses (it has not been observed for HIV-1 [276]) was initially interpreted to suggest that genetic recombination is not a routine process but instead is biphasic, with crossovers being either frequent or else nonexistent (68). It was thought that the performance of one recombination event predisposed a virus to performing additional switches or that only a subset of virions was prone to generating recombinant products due to flaws in viral nucleoprotein complex architecture (15, 133, 152). However, biases in gRNA packaging remove the need to evoke such models and suggest that recombination occurs at a similar high frequency for many if not all retroviruses (50, 221, 240, 368). This can also explain differences between MLV and HIV-1 in minus strong-stop switching. This replicative switch appears to occur essentially only between the two ends of a single RNA for gammaretroviruses but can occur either between ends of one RNA or from one to another for HIV-1 (318, 331, 355).

Other means of RNA copackaging. Patch repair by endogenous retroelements, which are unlikely to heterodimerize with gRNAs, was reported for simple retroviruses, as has recombination with RNAs that are likely encapsidated only occasionally and by chance (211). For example, experimental reversion of murine AIDS retrovirus *gag* point mutations revealed recombination with endogenous viruses and not point reversion in each instance (139). Endogenous retroviruses contribute less to HIV-1 genetics because no human retroelements resemble HIV-1, and gRNAs of human endogenous viruses are not readily packaged by HIV-1 (357). However, rarely encapsidated RNAs can contribute to recombination in proportion to their prevalence (12). In addition to the "random" low-level mRNA packaging, many RNA sequences that presumably cannot heterodimerize with gRNAs are capable of directing RNA packaging in a virus species-specific manner (62, 84). RNAs that become encapsidated but lack packaging and *cis*-acting signals can function as recombination substrates during provirus synthesis (120, 305).

FACTORS THAT MODULATE RECOMBINATION

Retroviral recombination does not occur at a uniform frequency, and the probability that template switching will occur

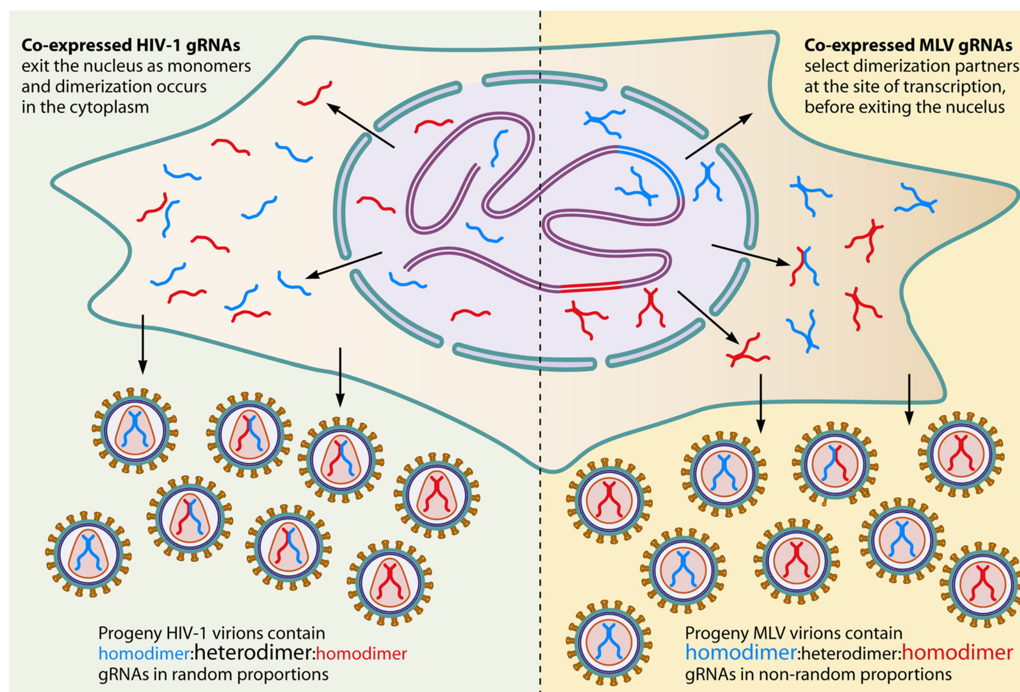

FIG. 10. Differences between gammaretroviruses and HIV-1 in gRNA selectivity. Shown is a schematic illustration of a single virion-producing cell coinfected with two distinct proviruses, shown as the double-stranded red and blue lines embedded within purple host DNA. Fates of gRNAs during HIV-1 assembly are indicated on the left, and those for the gammaretrovirus MLV are indicated on the right. MLV gRNAs preferentially self-associate at transcription sites in the nucleus, resulting in virions containing mostly homodimerized gRNAs. For HIV-1, RNAs dimerize at random, presumably in the cytoplasm, generating virions with a Hardy-Weinberg distribution of homodimers and heterodimers.

at a given template position is affected by enzymatic properties of RT, the availability of nucleotide substrates, and template structure and quality. This section describes factors that can increase or decrease recombination during HIV-1 replication. Interestingly, whereas HIV-1 displays more promiscuity in gRNA copackaging than simple retroviruses like MLV, and thus, its packaging properties make HIV-1 more recombinogenic than MLV, HIV-1 replication is somewhat less sensitive to environmental deficiencies that increase MLV recombination rates.

Intracellular Environment

Like all DNA polymerases, RT requires nucleotide substrates, and when nucleotides drop below the concentration required for maximal elongation, DNA synthesis slows. One difference between immortalized and primary cells is that the former are metabolically highly active. HIV-1 reverse transcription takes longer and/or is disrupted in quiescent cells (33, 356) and likely takes 20 h or longer in many clinically relevant cell types (260). Substrate limitation may not be the only restriction to viral replication in quiescent cells, but at least under some conditions, adding nucleosides is sufficient to restore DNA synthesis rates.

When MLV DNA synthesis is slowed by serum starvation or deoxynucleoside triphosphate (dNTP) pool imbalance, recombination rates increase dramatically (257, 310). Other environmental variables, such as reduced temperature, also increase template switching (190). One report claimed that HIV-1 recombination rates were higher in terminally differentiated cells than in actively dividing ones and that recombination might occur 30 times or more per replication cycle in certain cells in vivo (189). However, subsequent work that used more sensitive approaches to compare recombination in macrophages and T cells revealed no difference (51), and the treatment of HIV-1-infected cells with either hydroxyurea or AZT only modestly stimulated template switching (9, 233).

The main reason why HIV-1 recombination is less sensitive to dNTP concentrations than is MLV recombination probably reflects RT properties. Depending on the nucleotide, HIV-1 RT displays a 6- to 121-fold-higher affinity for dNTPs than does MLV RT, and thus, HIV-1 can recruit enough dNTPs to maintain maximal elongation rates even in cells with relatively small nucleotide pools (146). It has been suggested that the ability of lentiviral RTs to efficiently synthesize DNA in low-dNTP environments may be critical to replication in nondividing cells (82, 147). Consistent with this notion, HIV-1 RT active-site mutants with reduced dNTP binding affinities template switch more frequently than does wild-type RT (105, 241).

RNA Quality and Template Features

Template features that affect RT elongation likely influence recombination. Pausing contributes to template switching in part by allowing time for RNase H-mediated template strand cleavage and acceptor template access to nascent single-stranded DNA (26, 104). There is some evidence that template features that increase RT pausing in purified reactions promote recombination during viral replication (182). RNA sec-

ondary structure, homopolymeric stretches, and sequence context appear to modulate recombination (76, 89, 126, 191, 227, 248, 266). RNA structures also promote nonhomologous recombination (26, 86). Dissection of one recombination hot spot led to the conclusion that both RNA structure and extended donor-acceptor homology enhanced recombination (102). By analogy to *rho*-independent terminators of *Escherichia coli* transcription, it is fairly easy to conceptualize how RNA structure in a donor template might pause RT and promote recombination. However, some recombination hot spots have been linked to acceptor template structures (124, 219). At least in vitro, both structured and unstructured regions that do not encode detectable pauses can promote template switching (77, 224).

RNA breakage likely promotes recombination. It has long been noted that when scientists purify genomes from retroviruses, the RNAs are far more fragmented than are those from other RNA viruses (68). In light of the long history which retroelements have shared with their hosts and the growing list of host antiviral mechanisms, it is tempting to speculate that that the RNA smearing that retrovirologists observe on their gels may reflect some host innate antiviral activity and that part of the reason why retroviruses copackage two RNAs is to overcome this assault. Although one study that was designed to test the correlation between gRNA damage and gammaretrovirus recombination rates did not find striking enhancement, the results were likely influenced by the nonrandom segregation of gammaretrovirus gRNAs described above (134).

Breakpoint Clustering: Recombination Hot Spots or Products of Selection?

A weakness of assays that use crossover frequencies in a defined interval to deduce genome-wide recombination rates is that they assume that recombination occurs at uniform frequencies rather than principally at specific sites. Although recombination is observed throughout HIV-1 genomes and is roughly proportionate to length, frequencies are not uniform, and "hot" (or at least "warm") and/or "cold" spots for recombination do exist (14, 89, 148, 189, 346, 367).

Recombination breakpoint clustering in clinical strains is most often observed at the borders of viral genes (316). One recent study examining the breakpoints in clinical intersubtype recombinants found a frequent use of genetic intervals flanking the *env* gene (92). These did not appear to be dictated by homology, RNA structure, or regions implicated by experimental studies or toward the acquisition of sites under positive selection. Instead, some selection for replacing the entire *env* coding region appeared. Reported similar crossovers in HIVs propagated in vitro suggested that these are bona fide crossover hot spots, since cultured cells lack fitness determinants such as acquired immune responses (266). However, even subtle epistatic interactions can bias outcomes and favor parental alleles if, for example, one mutation's fitness varies depending on whether or not distal mutations are present, as predicted for residues that interact in a folded protein (184). Consistent with this notion, systematic analyses of clinical recombinants to identify positions where breakpoints are underrepresented suggest that only a small fraction of all recombinants generated survive within patient populations (17). Similarly, low infectivity has been observed for many chimeric envelope genes generated in vitro (180). Thus, most common crossovers observed in vivo are more likely the result of selection rather than template-switching hot spots. Similar pressures, layered over differing selective pressures in vivo, manifest themselves in ways such as selection for clustered compensatory changes that accompany mutations required for immune escape from specific cytotoxic T-cell responses (5, 164, 204).

One large-scale retrospective analysis of fitness for (primarily antiretroviral-treated) patient-derived *pol* sequences surprisingly concluded that, counter to theories on the benefits of sex and recombination, positive epistatic interactions outpaced negative ones in HIV-1 (34). Negative epistasis refers to the situation where two mutations together are more deleterious than either one alone (e.g., mutations amplify one another), and positive epistasis refers to situations where a second mutation counteracts the first (e.g., mutations antagonize one another). These conclusions, however, remain controversial, with some debate about data sampling and how fitness should be defined (340).

Sequence Similarity and Recombination Rates: Homologous and Homeologous Recombination

One factor that undeniably affects recombination frequency is the extent of sequence similarity between templates. The fundamental nature of nucleic acid base pairing means that sequence recognition occurs via complementarity and not identity. All models for retroviral recombination include steps where acceptor templates are recognized due to their complementarity to nascent DNA, and recombination rates are at least roughly proportional to the length of sequence identity (9, 155, 362). Thus, it is not surprising that enhancing homology enhances retroviral recombination.

The term "homeology" describes low-level sequence variation that lies somewhere between identity and nonhomology, as is the case when HIV-1 subtypes are compared. The effects of systematically various extents of sequence similarity on HIV-1 recombination have been examined using a repeat deletion assay (8). A 5% difference decreased the deletion frequency to 65% of that for identical repeats, with recombination declining further as more variation was introduced. When repeats differed by 27%, recombination was below the detection threshold of the assay, suggesting that switching was reduced more than 300-fold (8). These results show that HIV-1 recombination is somewhat less sensitive to genetic differences than is cellular DNA recombination (294, 339).

In the assay described above, sequence variation was artificially introduced and evenly distributed. In contrast, selection for maintaining essential features in viral genomes dictates that naturally arising variation can be more clustered. Examinations of crossovers in infectious virus in culture revealed that localized homology was a key factor (21, 22, 197, 214). Previously reported recombination between significantly different strains may arise essentially by chance, may rely on residual homology or homology-independent recombination triggers, or may be disproportionately common in short regions of high donor/acceptor template sequence identity (249, 265, 316). Small patches of sequence identity are often observed at otherwise-nonhomologous recombination junctions, suggesting that at

least some nonhomologous recombination is driven by highly localized regions of homology (361).

Nonhomologous Recombination

Retroviruses perform nonhomologous recombination about 1 to 0.1% as frequently as they perform homologous recombination (361). Nonhomologous recombination leads to HIV-1 length variation and is the likely mechanism of oncogene transduction by animal retroviruses (307, 345). Nonhomologous recombination can generate HIV-1 genetic variation that is important on the population level when it provides a selective advantage. If roughly five crossovers occur during every cycle of HIV-1 replication, then a 1% occurrence rate means that up to a few percent of all proviruses may contain one such nonhomologous crossover.

Most nonhomologous recombination likely leads to defective proviruses. However, even these can contribute to virus evolution, as explored further below. Specifically, the interaction of HIV-1 with its host counterintuitively provides some selection for the perpetuation of defective genomes (287). Although the half-life of most infected cells within a patient is brief, the failure to eradicate HIV-1 and the reemergence of archived strains attest to the persistence of HIV-1 and the opportunity for allelic reappearance by recombination.

Deletions, duplications, and insertions in deletions. The most common substrates for nonhomologous recombination are discontiguous portions of HIV's own genome. As shown in Fig. 11A, homologous recombination involves switching between the same locus on each of two copackaged gRNAs. In contrast, a switch from one gRNA to a position more 5′ on the copackaged gRNA will generate a deletion, while switching to a 3′ position will generate a duplication (Fig. 11B and C). Deletions generally cause defects, and indeed, many proviruses found in patients contain deletions (287). In some animal retroviruses, duplications—including enhancer duplications in the long terminal repeats of feline leukemia virus, or equine infectious anemia virus *env* variable region duplications to evade neutralizing responses—are important for pathogenesis (229, 365).

Experimentally, many nonhomologous crossovers display a pattern called "insertions in deletions" (87, 245, 248). Dissection of their structures led to the suggestion that they result from serial nonhomologous recombination and the propensity of the reverse transcription machinery to transfer to regions of microhomology in the absence of more extensive donor/acceptor identity (Fig. 11D). Once one such junction has formed, additional nonhomologous switches may be necessary in order to generate a provirus, and it is likely that many abortive reverse transcription products are generated for each double nonhomologous recombination insertion-in-deletion product. Thus, the remarkably high frequency with which inserts are observed within nonhomologous recombination junctions (>50% according to one study [87]) suggests that nonhomologous recombination may occur at a far-higher frequency than is detectable in intact proviruses. In this context, it is interesting to consider the potential of the recombination process to serve as an antiviral target. Some efforts to inhibit template switching—albeit without strong leads—have been reported

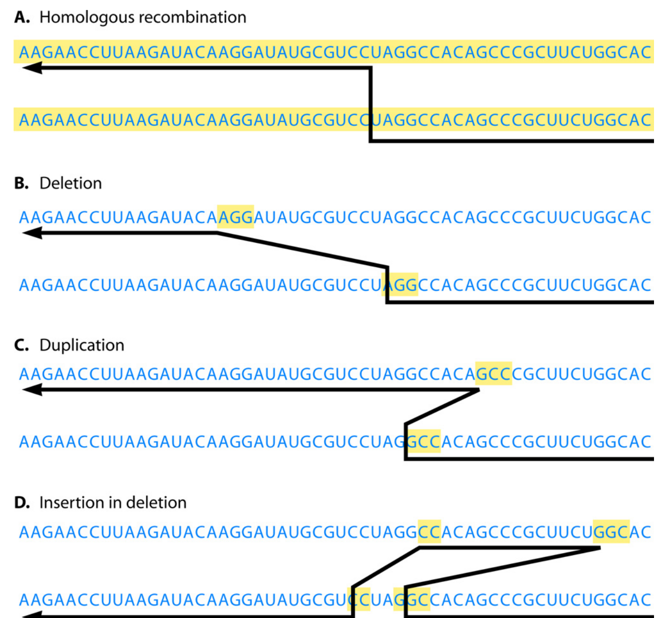

FIG. 11. Retroviral homologous and nonhomologous recombination. (A) Homologous recombination during HIV-1 DNA synthesis. Template switching occurs from one RNA to colinear identical sequences on the second RNA strand. (B to D) Microhomology-guided nonhomologous recombination during reverse transcription. (B) Template switching to a position more 5′ on the acceptor template to generate a deletion. (C) Template switching to a position more 3′ on the acceptor template to generate a duplication. (D) Insertion in deletion, or ectopic duplication, generated by template switching to the acceptor at a more 3′ position, continued synthesis, and a second template switch back to the donor strand at a more 5′ position. The arrows show the direction of DNA synthesis on two copackaged gRNAs. Shaded boxed sequences are regions of homology between donor and acceptor templates.

(74, 75, 324). However, it seems possible that enhancing or relaxing the specificity of template switching might have as great an antiviral effect as its outright suppression.

The insertions observed for nonhomologous recombination junctions are generally short (one study of nearly 70 inserts found that sizes ranged from 1 to 280 bases, with a median length of about 60 bases [87]). Such an "ectopic duplication" of viral sequences caused one reported drug resistance-associated mutation (194), and sequence analyses suggest other instances of ectopic viral sequence insertions (314, 329). The development of improved computational approaches for identifying transposed genome segments would likely reveal ectopic duplication to be a common means of lentiviral insert generation.

Oncogene transduction. Another form of nonhomologous recombination is host sequence transduction (360). Models for oncogene capture by animal retroviruses envision integration by chance upstream of a cellular oncogene and "leaky" polyadenylation signal readthrough to allow oncogene encapsidation as a gRNA 3′ appendage (311). Nonhomologous switching between the retrovirus and host sequences in the extended 3′ tail would generate a prototypical acute transforming virus (307) (Fig. 12). The vast majority of acute transforming retroviruses are replication defective, with the oncogene-containing genome being transmissible only during mixed infection with a replication-competent virus. A defective retrovirus that relies

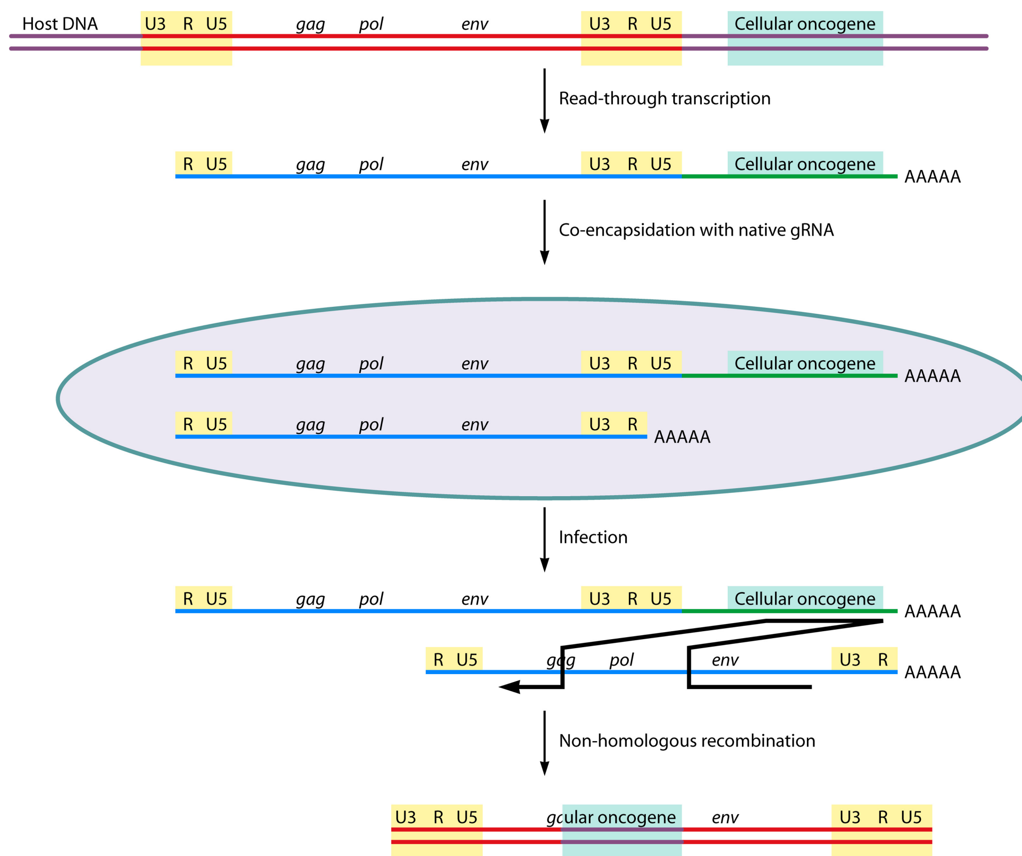

FIG. 12. Model for host oncogene transduction by animal retroviruses. After a rare chance event leads to the integration of a provirus DNA upstream of a cellular oncogene, readthrough of viral polyadenylation signal transcription can result in the "capture" of downstream oncogene sequences near the 3′ end of a readthrough RNA. Following packaging into a virion and infection of a fresh cell, this readthrough RNA can serve as a nonhomologous recombination template during reverse transcription. In most cases, oncogene transduction results in a replication-defective virus (307).

on complementing functions can, in some instances, become replication competent by recombining with its replication-competent "helper." In fact, there is some evidence that Rous sarcoma virus, possibly the only naturally arising replication-competent retrovirus containing a host oncogene, was replication defective initially (203).

There are no reports of human cancer caused by HIV-1 transduction of host oncogenes and few if any well-supported cases of insertional oncogene activation (296, 336). Why is this? HIV-1 infection is associated with several forms of malignancy, and much more clinical data are available for HIV-1 than for retroviral erythroleukemia in chickens or feline leukemia in cats (213, 229). Early beliefs were that AIDS patients died too soon to develop cancer, but it is now clear that many non-AIDS-defining cancers—essentially all common cancers except prostate cancer—are more prevalent among HIV-positive people than in the general population (246).

The reason why HIV-1 does not cause cancer by the mechanisms employed by simple retroviruses likely lies in differences in both replication and infection properties. For example, HIV-1 transcription and RNA trafficking rely on accessory factors whose expression or *cis*-acting signals might be eliminated by host sequence replacement of virus sequences. Even if such a gRNA were mobilized by a superinfecting virus, oncogene expression could not occur in the newly infected cell. If viral antigens *were* expressed, immune responses would eliminate the cell before a tumor could form. Thus, although the rescue of a defective HIV-1, initially by complementation and subsequently by recombination, has been described for virus in cell culture, the short half-life of infected cells in vivo may limit opportunities for replication-defective virus spread (143).

Short patch host sequence insertion. Although whole-gene transduction is very rare, the short inserts in nonhomologous recombination junctions are often host derived (87, 120, 121, 174). If HIV-1 polyadenylation signal readthrough occurred (as has long been recognized to be possible [19, 38, 81]), and a readthrough RNA were encapsidated (the packaging of lengthy HIV-1 gRNAs was also demonstrated [177]), then host sequences could provide HIV-1 length variation using the ectopic duplication mechanism shown in Fig. 11D.

The possibility that some of the length variation observed for HIV-1 might result from human sequence transduction is a rather old notion that has recently gained support (66). Length variation—sequence insertions or deletions—is surprisingly common in lentiviruses and associated with some forms of immune evasion, drug resistance, and long-term nonprogression (59, 167, 201, 262). For example, insertions in HIV-1 RT, sometimes but not always the result of local sequence duplication, can contribute to drug resistance (141, 345). Length variation is common in *env*, where an alternating occurrence of

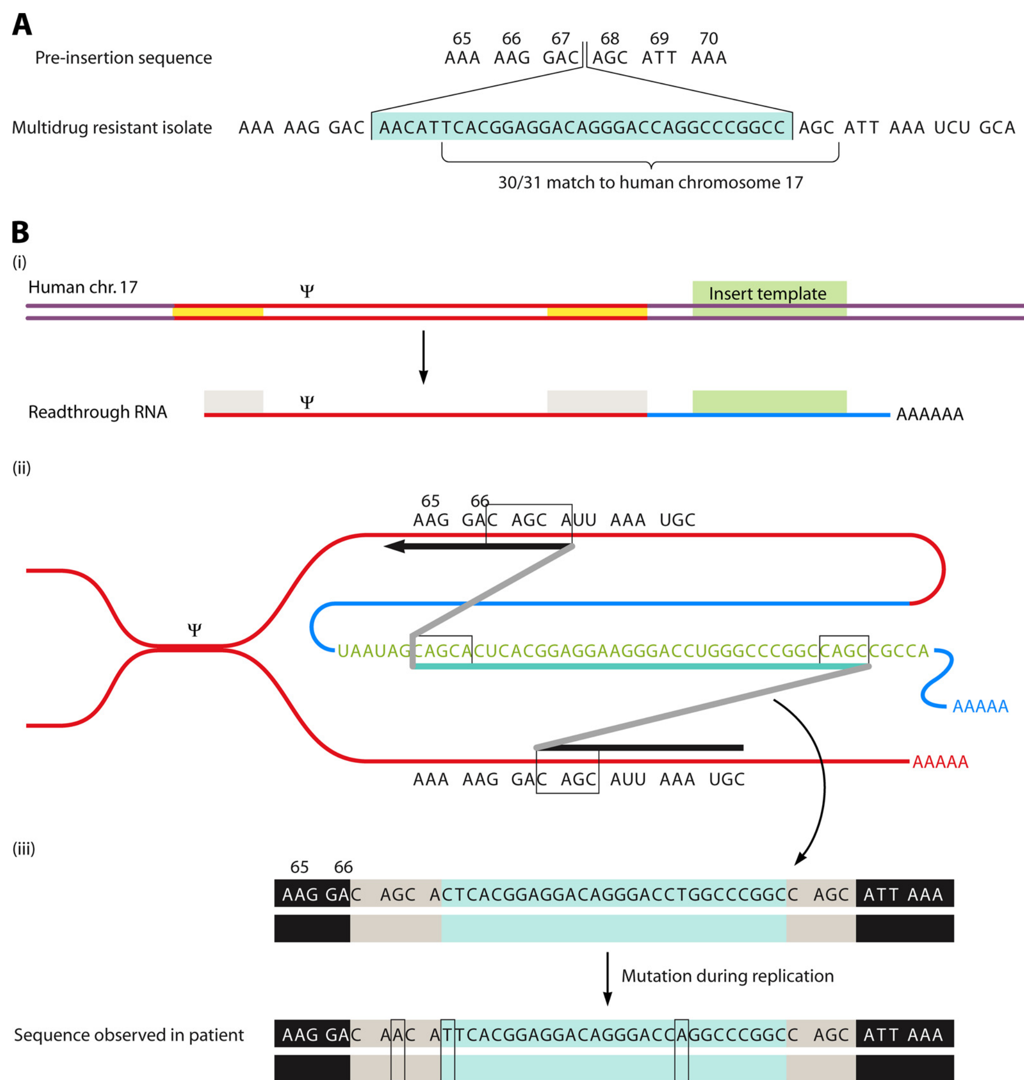

FIG. 13. Augmentation of HIV-1 multidrug resistance via human sequence transduction. Shown is a schematic overview of the mechanism by which an HIV-1 strain gained enhanced resistance to multiple RT inhibitors during replication in a Japanese child. (A) The top line shows a preinsertion sequence of the HIV-1 strain replicating in the boy's parents and in the child prior to the acquisition of drug resistance; the lower line indicates the sequence of the multidrug-resistant isolate. Blue shading indicates the region of a 30/31 match to human chromosome (chr.) 17. (B) Proposed mechanism for the acquisition of human sequences. (i) Establishment of a provirus upstream of the transduced sequences and generation of a readthrough RNA. (ii) Nonhomologous recombination between viral and human sequences. (iii) Further point mutagenesis during replication in the patient to generate the sequences observed for the patient (314).

insertions and deletions of variable region length has been observed during patient-to-patient passage (57, 193, 223, 363) and may contribute to immune evasion or coreceptor switch (238, 283). The appearance of shorter *env* variable regions upon transmission suggests that insertions are disadvantageous in newly infected individuals, as nonselected information is rapidly lost during retroviral replication (337).

An RT multiple-drug-resistance-augmenting insertion in an HIV-1 isolate from a Japanese child exemplifies short host sequence transduction (289, 314). Originally described as being a foreign 11-amino-acid insertion, this mutation's nonidentity with flanking sequences demonstrated that the 33 nucleotides were not inserted via the duplication mechanism described above (289). Marked differences in the G-C content (67% in the insert versus <40% for HIV-1 [28]) were consistent with possible horizontal gene transfer. By using BLAST to query GenBank with the insert plus flanking sequences revealed that its closest match, 30/31 bases of identity, was to the human genome. Molecular epidemiology and mechanistic modeling demonstrated that the insert likely resulted from nonhomologous recombination between virus and host sequences, followed by additional mutagenesis, as described in Fig. 13 (314). These findings provide evidence that human sequences are, at least rarely, transduced into HIV-1 genomes, where they can contribute to genetic and phenotypic variation. As the example here shows, once acquired, the ancestry of transduced sequences may be obscured by selection for functionally optimized variation.

The use of the human genome to query HIV-1 isolates readily identifies insertions assigned low BLAST expect scores (which are functionally equivalent to *P* values and indicate the

unlikelihood of chance correlation). Consistent with predictions of chance among large correlation sets (e.g., Bonferroni's correction) and despite their significant BLAST scores, some of these insertions likely do not represent transduced sequences. Additional matches so identified may reflect the inadvertent introduction of host sequences into databases by investigator error. However, one prominent class of insertions that often scores as significantly similar to host sequences is *env* variable region inserts (12). These N-linked glycosylation site-encoding regions in HIV-1 isolates have a G-C content very different from those of other HIV-1 genome regions and recede and reappear episodically rather than by gradual point mutation (35, 170, 363) (Fig. 14A). Some are duplications of adjacent sequences likely generated by the mechanism shown in Fig. 11C. However, some, including SIV *env* extensions that arise in experimentally infected animals and do not exist in the virus that initiates infection, do not match flanking sequences (242) (Fig. 14B). Instead, the sequences encoding these O-linked glycosylation targets, like the N-linked sites in HIV-1, bear strong resemblance to primate genomes' microsatellite repeats (306, 366). Provocatively, one such insert—with 30/30 bases of identity between the HIV-1 isolate (GenBank accession number AF530576) and human chromosome 3—appears to lie at a junction between subtypes in a new circulating recombinant (326) (Fig. 14C). This pattern is suggestive of the "insertions in deletions" that are commonly observed experimentally.

The model for generating host insertions in *env* variable regions is as follows. First, a provirus is established upstream of a host region containing microsatellite repeats. Although largely a chance event, the high density of these repeats in the human genome makes this event less rare than insertion near a specific locus. Following viral polyadenylation readthrough and readthrough RNA packaging, nonhomologous switching between *env* sequences and microsatellites in the RNA's 3′ extension would patch in the host sequences. This rare event might be stimulated by microhomology retained when a viral ancestor deleted a previous insert.

Proposing that host sequences contribute to HIV-1 variation requires evoking a series of rare events, but other virus systems provide a compelling precedent. Such rare events are well-established contributors to the pathogenesis of several animal retroviruses (211), and phenotypic switch due to host sequence transduction is known to contribute to pathogenesis in even more distally related viruses such as bovine pestiviruses (24, 27, 333).

Studies of bacteria indicate that successful gene transfer between species in nature depends on factors such as the ecological isolation of the species and the recipient cell's ability to recognize and remove foreign sequences. For retroviruses, which replicate in intimate association with their cellular hosts' genomes, these factors are clearly far less of an impediment to gene transfer than they are for bacteria. The activity of the recombination machinery is also critical to horizontal transfer, and this machinery is especially robust for retroviruses like HIV-1. Also important to the cross-species transmission of genetic material is the fitness of the transductant. Strong examples of host sequence insertions, such as the multidrug resistance-associated changes described above, demonstrate that host sequence transduction contributes to selectively advantageous HIV-1 variation at least rarely (Fig. 13). The properties of *env* variable regions suggest that host sequence transduction may contribute to some common forms of HIV-1 length variation as well.

Recombination with Unlinked Sequences

The model for transduction described above suggests that host sequences gain access to the recombination machinery via readthrough gRNAs (307). However, other means of host sequence recruitment may also contribute. As mentioned above, host mRNAs are encapsidated by HIV-1 in rough proportion to their intracellular levels, and many nonmessenger RNAs are also encapsidated (284). One study that revealed host sequence transduction during HIV-1 vector replication showed that inserted sequences were not packaged on readthrough gRNAs (309). However, the sequences were present in GenBank expressed sequence tags, suggesting that they were part of a human mRNA that was randomly copackaged (309).

RECOMBINATION AND THE NATURAL HISTORY OF HIV-1

Recombination contributes to HIV-1 genetics on several levels: from the origins of the virus to its adaptability in individual patients (208, 265, 268, 323). The chimpanzee virus that gave rise to HIV-1 was likely a recombinant of other SIVs (129). Phylogenetic analyses suggest that the rare HIV-1 group N arose via recombination between an SIV and an early form of group M (103). It has been suggested that molecular clock-based estimates of the timing of primate lentivirus transmission to humans may be off due to recombination (267). Within individual patients, recombination is an extensive and ongoing source of viral diversity (47).

Recombination over the Course of HIV-1 Infection

The genetic breadth of quasispecies within individual patients varies over the course of infection. Although patients differ—for example, heterosexually infected women typically display high virus diversity in early infection—a significant genetic bottleneck is generally observed upon transmission. This is followed by a period of years during which viral divergence includes adaption to host immune pressures and a fairly steady accumulation of neutral changes balanced by some evolution toward the root of the patient's viral phylogenetic tree (223).

Within individual patients, viruses become compartmentalized and subpopulations adopt independent evolutionary pathways. Thus, the amount of genetic information available for recombination (the effective population size) is much less than that on the whole-infected-person scale (297). Intrapatient variation in the nature of reservoirs has been reported. Differing selective pressures likely contribute to genetic variation among compartments' HIV-1 populations and may limit how much of the genetic heterogeneity within individual patients can serve as sources of intrapatient recombination (209, 258).

Latent Reservoirs and Recombination in HAART-Treated Patients

An important requirement for recombination is the amount of diversity available. Recombination does not occur in nonreplicating virus and, all else being equal, is proportionate to the amount of virus replication. Consistent with this, the more

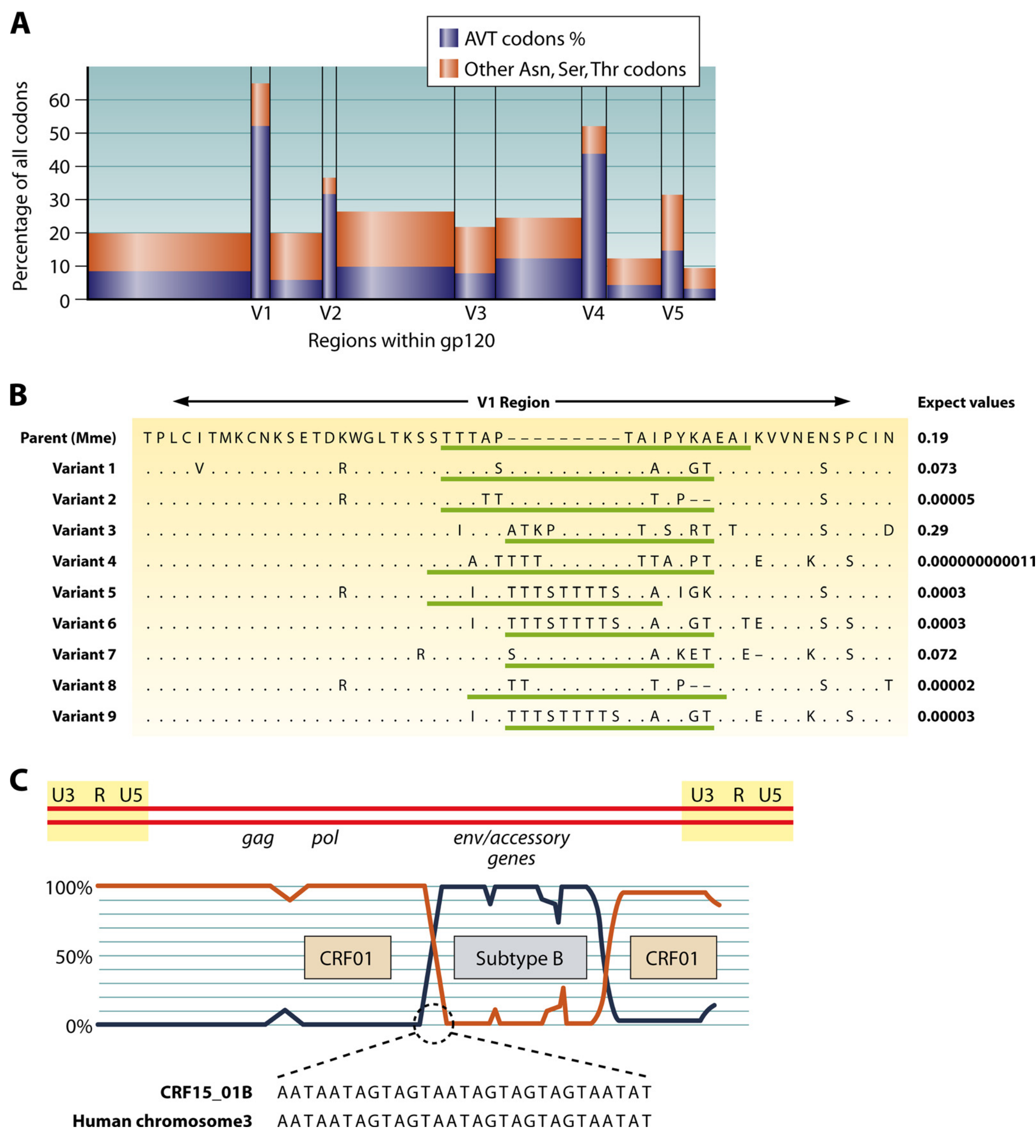

FIG. 14. Candidate host sequence insertions in lentivirus genomes. (A) HIV-1 AVT codon-rich *env* variable region insertions. (Based on patient *env* sequence data as analyzed by in reference 170.) The *x* axis indicates boundaries of genetic segments within the gp120-encoding portions of *env*. Blue bands indicate proportions of all codons within each genetic region that are AVT (where V is A,C, or G) codons for Asn, Thr, and Ser; orange bands indicate proportions of all codons that are other Asn, Thr, and Ser codons. Note that the most prevalent AVT codon observed in HIV-1 variable regions, AAT, is also the most common trinucleotide repeat in human microsatellite DNA (306). (B) Length variation that emerged in macaques infected with an SIV molecular clone (Based on data presented in reference 242.) Shown are V1 region amino acid sequences for the parental Mne strain (GenBank accession number M32741) and the nine variant classes reported after the development of AIDS-like symptoms (accession numbers M79283, M79284, M79287, M79288, M79286, M79289, M79285, M79299, and M79293). Nucleotide sequences were used in unfiltered blastN to query all macaque sequences in GenBank. As indicated by the expect values (E values are similar to *P* values) presented at the right, length variation in all but one variant (variant 3) significantly increased the similarity of that SIV strain to macaque sequences under stringent search parameters (+1/−4 match/mismatch penalties), suggesting that these inserts, which lack significant similarity to the parental Mne genome, were host derived. (C) Host-like insertion between sequences derived from two different subtypes in a new HIV-1 CRF. (Based on data presented in reference 326.) The figure indicates the genome positions where bootstrap analyses indicated breakpoints between subtypes in CRF15_01B. The dotted circle and lines and aligned sequences at the bottom indicate the location of a region of 30 bases of identity between this HIV-1 isolate (GenBank accession number AF530576) and human chromosome 3 (accession number NT_005612.15), which is assigned an expect value of 0.001 in unfiltered blastN with default search parameters. Note that this structure is reminiscent of the host-derived inserts that frequently splint experimentally derived nonhomologous crossovers (87).

dramatic a drop in virus load observed upon drug treatment, the less recombination contributed to rebounding populations (171). Thus, undoubtedly less recombination occurs for patients whose virus replication is effectively contained than for patients where replication proceeds unchecked.

Although highly active antiretroviral treatment (HAART) can reduce viremia to below levels detected by standard tests, more sensitive methods reveal very low levels of viremia, and no one has been cured of HIV-1 infection. Thus, much current effort is being devoted to understanding the nature of persis-

tent viruses and how or if these viruses can be eradicated. The classic view of latency is cells in which HIV-1 persists as a stable but nonexpressed provirus (123). There is also evidence for some patients of a prolonged production of viruses from a limited number of infected cells without the evolution that would accompany cell-to-cell spread (20).

The relative contributions of continuous low-level virus spread versus persistence in a largely quiescent state has been debated for several years. The bulk of current data, which suggest that virus of patients on HAART is archival and not accumulating genetic changes, suggests that most residual virus is released from stable reservoirs: cells that are reactivated or can somehow, at least on a population level, continue to release virus years after initial infection (293). Because recombination requires the completion of a round of replication and because the drugs in treatment cocktails specifically inhibit replication steps (e.g., reverse transcription), strict HAART adherence should severely curtail, if not outright eliminate, allelic reappearance via recombination. However, data from phylogenetic studies of the resistant strains that emerge during changing treatment regimens confirm the importance of recombination to rapid adaption under conditions of changing drug selection (235).

Recombination and the Implications of Proviral Persistence

Within infected people, the half-life of most productively infected cells is roughly 2 days (252). This rapid clearance of cells expressing viral antigens provides selection for the maintenance of defective proviruses and the high proportion of defective genomes among patients' integrated virus population (292). Viral genome inactivation is more frequently the result of a single lethal event rather than the cumulative effects of sequential sublethal events (308). Any proviral defects that shield infected cells from immune detection should have a survival advantage (43). Thus, it is likely that most viral relics contain significant segments of functional genetic material. Because two different defective retroviruses can recombine to form infectious virus, even defective genomes can contribute to the reemergence of dormant genetic information. Intrapatient recombination between circulating strains and archived proviruses contributes to the dynamics of drug resistance and may contribute to the evolution of the coreceptor switch as well (47, 118, 212, 235, 271). It is likely that the extent of recombination between variants that arise within an individual has been underappreciated because of the similarity of parental strains (117).

Similar findings have been observed for tissue culture. Some cells used as models for latency harbor defective proviruses, including *tat* mutations in the U1 cell line (90) and various accessory gene mutations in other persistently infected cells (169). The resurrection of infectious virus-containing alleles from these inactive "latent" proviruses via recombination with superinfecting viruses has been reported (168).

Defective genomes dominate the provirus population in patients where replication is controlled by immune responses or antiviral therapy. Thus, notions that deleterious mutations will be lost from the gene pool, which pertain for cellular organisms and even for most viruses, do not apply strictly to HIV-1. Strains isolated from patient RNA versus patient integrated DNA show that the RNA represents actively replicating virus, whereas most proviruses are at least 6 months old, many are defective, and most are from cells not expressing viral RNA (292). Whereas negative selection does not eliminate defective variants, as it would for other RNA viruses, a form of "survival of the fittest" is nonetheless at play, if this is taken to mean "survival of the survivors." For such patients, the bulk of the HIV-1 DNA-level persistence landscape might best be described as "survival by death," where being defective is a form of fitness (287).

This property of HIV-1 might limit the promise of strategies to eliminate HIV-1 by lethal mutagenesis (16, 300). HIV-1 appears to replicate near the error threshold, with mutation rates and fitness being carefully balanced, thus allowing the virus to maximize its mutability and adaptive potential. As a result, some have suggested that HIV-1 might be eliminated by error catastrophe, which is a promising approach for the therapeutic elimination of other RNA viruses (83, 113). However, at least some of the experimental work in support of HIV-1 lethal mutagenesis has relied on the serial passage of cell-free virus, thereby removing defective proviruses from the genetic landscape (195, 317). Since one factor that makes recombination especially important to HIV-1 clinical diversity is the persistence of genomes as integrated proviruses, this leaves the prospects of HIV-1 lethal mutagenesis somewhat unresolved.

Observations related to the host restriction factor A3G, which introduces G-to-A hypermutation into HIV-1 genomes in the absence of Vif, shed some light on this prospect. The fact that HIV-1 evolved a factor whose principal role appears to be avoiding hypermutation seems to suggest strong selective pressure to avoid hypermutation. Furthermore, some work comparing patient samples suggested that A3G mRNA levels inversely correlate with viremia and progression to AIDS (149). However, others have suggested that countering Vif, and thereby enhancing A3G action, may not have the desired effects (261). Indeed, one study demonstrated that mutations associated with resistance to the RT inhibitor lamivudine arose via hypermutation in the absence of drug selection when naturally arising partially defective *vif* mutants were propagated. The recombination-mediated transfer of these mutations into replicating virus was observed under selective conditions, thus demonstrating that defective hypermutated genomes can contribute to virus evolution (222). A crucial caveat is that this work was performed using cultured cells. However, molecular epidemiology provides evidence of similar molecular patterns in drug-resistant transmitted patient viruses (109), suggesting that under strong selective pressures, recombination can lead to the acquisition of advantageous gene segments even from highly defective genomes. However, despite the above-described considerations that suggest that defective proviruses have the potential to allow HIV-1 to avoid the genetic meltdown characteristic of error catastrophe, the defective endogenous viruses littering our genomes provide evidence that retroviral extinction is possible.

CONCLUSIONS

The RT template switching that causes HIV-1 genetic recombination occurs at the remarkable frequency of about four or five times on average during the synthesis of every HIV-1

DNA. The vast combinatorial potential of HIV-1 genetic recombination presents one of the greatest challenges to preventing HIV-1 infection and combating HIV disease because it introduces genetic variation that complicates vaccine development and promotes escape from antivirals. Most recombinogenic crossovers occur without introducing errors, in regions of extensive sequence homology between the two gRNAs which each virion encapsidates. Some experimental work supports the notion that template switching is a replication fidelity mechanism that enhances the frequency of intact provirus synthesis. Rare nonhomologous recombination events can yield defective proviruses that contribute to the latent reservoir or can generate HIV-1 genome-length variation that is associated with viral phenotypic shift. The transmission properties of HIV-1, which lead to relatively frequent cell coinfection, paired with the way assembling HIV-1 particles recruit their gRNAs lead to unusually frequent copackaging of two different gRNAs. These properties, along with the high level of viral replication in infected people, present the potential for a vast network of genetic marker reassortants. Such recombinants have contributed to the genesis of the major strains now in global circulation, the evolution of virus strains in individual patients, and the very high level of genetic variation that has thus far thwarted containment of the pandemic.

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Adewunmi Onafuwa-Nuga was born and educated through high school in Nigeria. In 1999, Adewunmi received her bachelor of science degree (with honors) in biology from Concord University, Athens, WV. She then spent almost 2 years comparing recombination rates between HIV-1 and MLV in the laboratory of Dr. Alice Telesnitsky before beginning graduate studies at the University of Michigan. As a graduate student in Dr. Telesnitsky's laboratory, she focused on RNA packaging and recombination, and her research demonstrated that host RNAs packaged by retroviruses are selected from the intracellular pool in a nonrandom fashion. Adewunmi obtained her Ph.D. in microbiology and immunology in 2007. Adewunmi recently moved to the Internal Medicine Department at the University of Michigan and became a research fellow in Dr. Kathleen Collins' laboratory. She is currently studying HIV-1 infection of bone marrow progenitor cells.

Alice Telesnitsky is a fifth-generation Californian who was educated from kindergarten through her Berkeley doctoral studies in the California public schools. Following her graduate work on *E. coli* transcriptional elongation with Michael Chamberlin, she continued her interests in polymerase mechanisms through reverse transcriptase structure-function studies as a postdoctoral fellow with Stephen Goff at Columbia University in New York. Now in her 15th year as a faculty member at the University of Michigan, her laboratory employs genetic, computational, and biochemical approaches to studying reverse transcription as well as viral and host cell RNA properties that contribute to retroviral replication.