

Targeted DNA Mutagenesis for the Cure of Chronic Viral Infections

Joshua T. Schiffer,^{a,b} Martine Aubert,^a Nicholas D. Weber,^{a,c} Esther Mintzer,^{a,d} Daniel Stone,^a and Keith R. Jerome^{a,c}

Fred Hutchinson Cancer Research Center, Vaccine and Infectious Diseases Division, Seattle, Washington, USA^a; Department of Medicine, University of Washington, Seattle, Washington, USA^b; Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA^c; and Department of Microbiology, University of Washington, Seattle, Washington, USA^d

Human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV), and herpes simplex virus (HSV) have been incurable to date because effective antiviral therapies target only replicating viruses and do not eradicate latently integrated or nonreplicating episomal viral genomes. Endonucleases that can target and cleave critical regions within latent viral genomes are currently in development. These enzymes are being engineered with high specificity such that off-target binding of cellular DNA will be absent or minimal. Imprecise nonhomologous-end-joining (NHEJ) DNA repair following repeated cleavage at the same critical site may permanently disrupt translation of essential viral proteins. We discuss the benefits and drawbacks of three types of DNA cleavage enzymes (zinc finger endonucleases, transcription activator-like [TAL] effector nucleases [TALENs], and homing endonucleases [also called meganucleases]), the development of delivery vectors for these enzymes, and potential obstacles for successful treatment of chronic viral infections. We then review issues regarding persistence of HIV-1, HBV, and HSV that are relevant to eradication with genome-altering approaches.

Chronic viral infections cause enormous suffering among infected individuals, highlighting the need for curative therapies. Thirty-three million people worldwide are infected with human immunodeficiency virus type 1 (HIV-1), and the annual global incidence is approximately 2.6 million infections (186). With the advent of highly active antiretroviral therapy (HAART), HIV is now managed as a chronic rather than a terminal disease (108, 120) and treated patients have a normal life span (6). However, only 36% of those in need of antiretroviral regimens in low- and middle-income countries have access to therapy (186). Without treatment, the median time for progression to AIDS is 9 to 10 years and the average life expectancy upon developing AIDS is 9 months (113). Moreover, HAART does not cure the infected individual or reverse all disease manifestations, and therapy is lifelong. Other sobering facts include possible development of drug resistance and long-term adverse effects of current drugs, as well as the enormous financial burden of lifelong treatment in populations in which HIV is endemic (120, 183).

Hepatitis B virus (HBV) is another infection of enormous public health importance. Though an effective vaccine is available, global uptake is low (51, 100). Over 350 million people are chronically HBV carriers, and more than 50% of people within certain regions of Asia and Africa have a history of HBV exposure. Cirrhosis, liver failure, and hepatocellular carcinoma (HCC) related to HBV claim 500,000 to 1.2 million lives annually (36, 94). Antiviral therapy is effective for preventing these outcomes but does not eliminate all reservoirs of virus. Only a small fraction of the infected population has access to therapy, which typically must be given over years (209). Liver transplantation, an option for infected persons with end-stage disease, is unavailable for the majority of those in need.

Herpes simplex virus (HSV) is also a cause of significant morbidity. HSV-2 is the leading cause of genital ulcers worldwide, and 16% of Americans are seropositive (208). HSV-1 prevalence exceeds 50% in the United States: this serotype can cause both oral and genital ulceration and is the most common etiology of infectious blindness (keratitis) and viral encephalitis (158). Both varieties of HSV cause severe infections in newborns and immuno-

compromised hosts (17). Importantly, HSV-2 is a key risk factor for HIV-1 acquisition and transmission (163, 196). While antiviral therapy decreases the severity of primary infection and recurrent ulcer formation and also decreases the frequency of asymptomatic viral shedding and recurrences, it is imperfect for each of these indications (38). Several candidate vaccines failed to demonstrate efficacy (37).

Effective antiviral therapies exist for treatment of each of these infections, and development of these agents represents one of the major successes in medicine during the previous decades. Accordingly, the search for new antiviral medications continues to be a major focus within virology. Unfortunately, antiviral therapies may have limited room for improvement. While existing treatments for HIV, HBV, and HSV inhibit replication and cellular entry of the virus extremely potently (164), they do not target latent viral stores which exist in a reversible nonreplicating state of infection. HIV-1, HBV, and HSV establish long-lived reservoirs from which newly synthesized viruses can continually arise. When antiviral agents are stopped, robust viral replication often resumes, and symptomatic manifestations of disease typically follow.

Because antiviral therapy is safe and mostly effective for these infections, relatively little attention has been paid to approaches that might rid the body of latent virus. However, a deeper understanding of the molecular nature of latent viral genomes and their cellular and anatomic sites has raised the possibility that new therapies may directly attack the Achilles' heel of chronic viral infections. Recently, new technologies that may allow specific disruption of latent viral genomes have been developed. In this review, we outline why highly specific, DNA-cleaving enzymes, an exciting technology that was recently recognized as the "Method of the

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Address correspondence to Keith R. Jerome, kjerome@fhcrc.org.

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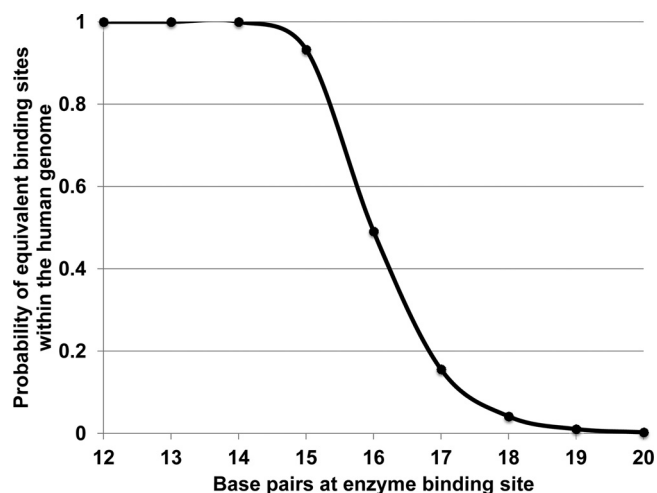


FIG 1 Probability of cDNA base pairs within the human genome according to the base pair length of the cleavage enzyme. The calculation assumes random ordering of nucleotides within the human genome.

Year” (110), may enhance the likelihood of a cure. We review the major classes of such enzymes and consider specific issues for their use in addressing the unique latent reservoirs for HIV-1, hepatitis B, and HSV-2.

SPECIFIC TYPES OF CLEAVAGE ENZYMES

For a DNA-targeting enzyme to be useful for gene therapy, it must possess adequate specificity for the target sequence such that it will not recognize off-target genomic sites. Therefore, enzymes need to target DNA stretches of sufficient length. Assuming homogeneous mixing of nucleotide sequences across the 2,900,000,000-bp human genome, the probability of a complementary sequence to a viral target site can be estimated according to the formula $1 - [1 - (1/4^x)]^{2,900,000,000}$, where x is the number of base pairs in the enzyme binding site. This suggests that the use of cleavage enzymes that target 17 or more nucleotides will minimize the likelihood of off-site binding (Fig. 1). (There are 4^{17} or 1.72×10^{10} possible 17-nucleotide sequences for an enzyme that targets a DNA sequence with overall specificity equivalent to that of an invariant 17-nucleotide region.) Factors such as sequence homology due to incorporation of ancient retroviral sequences into the human genome may enhance the probability of equivalent binding sites, while chromatin may impede access to complementary se-

quences within noncoding portions of the genome. Therefore, the extent of off-target binding is difficult to estimate precisely. Nevertheless, these considerations suggest that a DNA-binding enzyme of sufficient specificity might successfully target viral DNA while leaving the host genome intact.

Currently, 3 classes of DNA-recognizing/modifying enzymes with the required high degree of specificity are known: zinc finger nucleases (ZFNs), transcription activator-like (TAL) effector nucleases (TALENs), and homing endonucleases (HEs). Each class of proteins has unique features that are potentially advantageous and disadvantageous for eradication of chronic viral infection (Fig. 2).

Zinc finger nucleases. The zinc finger nucleases (ZFNs) are the most highly developed of the site-specific nucleases. DNA specificity results from the specific binding of tandem arrays of zinc fingers, each of which recognizes either a unique or a slightly degenerate DNA triplet within the context of the longer cognate target site (190). To achieve DNA cleavage activity, an array of zinc fingers is covalently tethered to the nonspecific nuclease domain from the R.FokI (FokI) restriction endonuclease. Since the FokI nuclease is active only as a dimer, two separately encoded, appropriately spaced zinc finger arrays must be designed to bind opposing half-sites of a desired DNA target, thereby facilitating cleavage at the desired site. In most systems, three or four zinc fingers are incorporated in each protein subunit. The outcome of ZFN activity is a DNA double-strand break with 5' overhangs, located between the zinc finger-defined DNA-binding motifs.

The ZFNs are attractive gene-targeting constructs since the DNA triplets recognized by many individual zinc fingers have already been defined. However, ZFN technology also has certain limitations (162). Zinc fingers targeting certain DNA triplets are not yet available, and thus, only a limited number of DNA sequences can be accessed. ZFN specificity can be influenced by context dependence, in which neighboring zinc fingers can alter either the recognition specificity or the affinity of a given zinc finger, further limiting possible targets. Zinc fingers can also have substantial binding affinity for sequences that are similar but not identical to their intended targets. When coupled with the non-specific DNA cleavage activity of the FokI nuclease, this can result in substantial off-target DNA cleavage and toxicity by some ZFN designs (68). Off-target cleavage has been reduced by the development of FokI variants that can function only as heterodimers, thus preventing cleavage by undesired homodimers (176), but questions remain regarding the ultimately achievable specificity of ZFNs. Despite these potential limitations, ZFNs have achieved

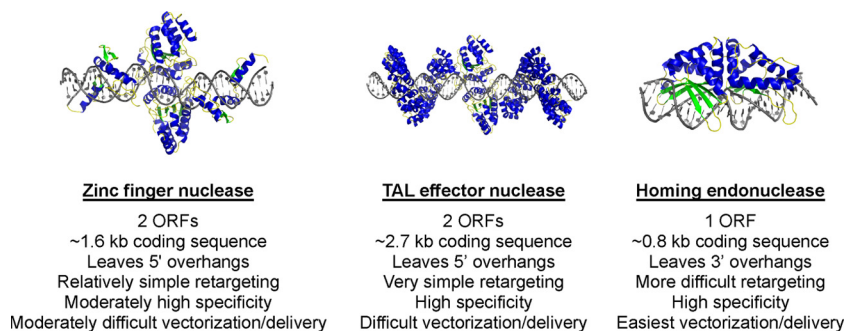


FIG 2 Structures of cleavage enzymes.

major successes, having been used extensively in the study of knockout of the HIV coreceptor CCR5 (75, 137, 204) and more recently to directly target HBV covalently closed circular DNA (cccDNA) in infected hepatocytes (39).

TALENs. The transcription activator-like (TAL) proteins were originally identified in certain plant-pathogenic species of *Xanthomonas* (reviewed in reference 13). The TAL proteins contain highly modular architectures that include an N-terminal region which interacts with the bacterial type III secretion system, a central region containing multiple tandem copies of a unique DNA-recognizing repeat domain, and C-terminal nuclear localization signal (NLS) and transcriptional activation regions. After being delivered from bacteria into plant cells and nuclei, these proteins specifically bind DNA targets within promoter regions of the plant genome, after which the transcriptional activation domain induces gene overexpression. The role that this process plays in bacterial pathogenesis in plants is currently an area of intense investigation.

The DNA specificity of the TAL proteins is mediated by a variable number (often 15 to 20) of tandem repeats of a 34-amino-acid repeat sequence. The repeats are nearly identical, with the exception of two variable amino acids at position 12 and 13, which comprise the repeat variable diresidue (RVD) motif (12, 114). The RVD motif specifies the DNA base recognized by each repeat domain, and the “code” of RVDs specifying each of the 4 DNA bases has been determined (12, 114). By combining the DNA recognition domains of the TAL proteins with the FokI nuclease, specifically targeted DNA cleavage enzymes (TAL effector nucleases [TALENs]) have been generated (28, 97).

The TALENs have certain advantages that make them particularly attractive constructs for specific DNA targeting. As noted above, each repeat of the DNA recognition domain mediates the interaction with one nucleotide. Furthermore, there is very little apparent context dependence between individual repeats. Thus, many desired DNA sequences can be targeted, and the resulting TALENs typically display DNA specificity and cleavage activity (24, 98).

The major drawback to the use of the TALENs is their large size. Each repeat of the DNA-binding domain (which recognizes a single nucleotide) is bulkier than an individual zinc finger (which recognizes a DNA triplet). The extremely large nature of these proteins may raise significant difficulties in vectorization and delivery, especially for *in vivo* use. Moreover, similar to zinc finger nucleases, DNA cleavage requires expression or delivery of two protein chains that subsequently dimerize the FokI nuclease domain at the site of cleavage.

Homing endonucleases. Homing endonucleases are an especially promising class of DNA-cleaving enzymes (173). In nature, these proteins are encoded by homing endonuclease genes, which are selfish genetic elements found within introns in certain microbial organisms, phages, and viruses. Homing endonucleases introduce DNA double-strand breaks within homologous alleles that lack the corresponding intron. These breaks are then repaired via homologous recombination using the allele containing the homing endonuclease gene, thus driving the selfish genetic element into the susceptible target.

Homing endonucleases are attractive as DNA-targeting enzymes for two main reasons. First, unlike the ZFNs or TALENs, DNA-cleaving activity is inherent to the same protein domains also responsible for DNA binding (note that the cleavage mecha-

nism of HEs results in 3' DNA overhangs, in contrast to the 5' overhangs resulting from the FokI nuclease used in ZFNs and TALENs). HEs are therefore significantly smaller (generally around 250 amino acids) than ZFNs or TALENs, thus facilitating vectorization and delivery. Second, HEs typically display extremely high specificity for their DNA targets (a property that presumably has resulted from long-term selective pressure to avoid fitness costs on their biologic hosts) and therefore theoretically will induce less off-target cleavage than ZFNs. Thus, many HEs show little if any toxicity in cells, even when expressed at very high levels for extended periods (8).

The major obstacle to widespread use of HEs has been the technical difficulties that are inherent in the process of redirecting their cleavage specificity to desired DNA targets. The DNA contact surfaces and residues of HEs do not possess the modular structure of either zinc fingers or the TAL proteins. Thus, their protein-DNA interactions and corresponding specificity tend to display significant context dependence that is strongly influenced by neighboring amino acids and DNA contacts. Furthermore, the association of DNA binding and cleavage activity within the same protein domain means that alterations in protein structure to facilitate DNA binding can have deleterious effects on the cleavage activity of the HE. Despite these obstacles, there have recently been several impressive achievements in redirecting HEs toward useful targets (7, 63, 185), and technical advances in HE redesign and targeting are occurring continually (173). Thus, the 3 classes of proteins (ZFNs, HEs, and TALENs) each have unique advantages and disadvantages, and the ideal protein may vary depending on the particular application envisioned.

EFFECTS OF DNA TARGETING AND GENE KNOCKOUT

The ability to induce DNA double-strand breaks at defined sites using ZFNs, TALENs, or HEs has important implications for gene therapy. DNA double-strand breaks are incompatible with continued cell viability, and thus, powerful DNA repair mechanisms are triggered. In mammalian cells, the presence of DNA double-strand breaks increases the frequency of homologous recombination by orders of magnitude (155, 171). This raises the possibility of targeted gene correction by the simultaneous delivery of a corrected DNA repair template along with the DNA-cleaving enzyme. This targeted repair would avoid dangers inherent in random insertional gene therapy and also ensures that the corrected sequence remains under the proper endogenous expression control elements (reviewed in reference 140). Such targeted repair has been used in a variety of mammalian cell types (reviewed in reference 143).

However, in mammalian cells, the predominant repair pathway for a DNA double-strand break is not homologous recombination but, rather, nonhomologous end joining (NHEJ). During NHEJ, the ends of the cleaved DNA are directly religated, and in most cases, repair is precise, resulting in restoration of the original DNA sequence (Fig. 3). However, in the presence of a targeted DNA endonuclease, precise repair restores the recognition sequence for the enzyme, leading to repeated rounds of cleavage and repair. The cycle repeats until an imprecise repair event, such as an insertion/deletion (indel) or more complex mutation, occurs. Once the recognition site for the enzyme is altered, the enzyme no longer targets the site. Cleavage enzyme-induced deletions and frame shifts within a DNA target can eliminate the synthesis of

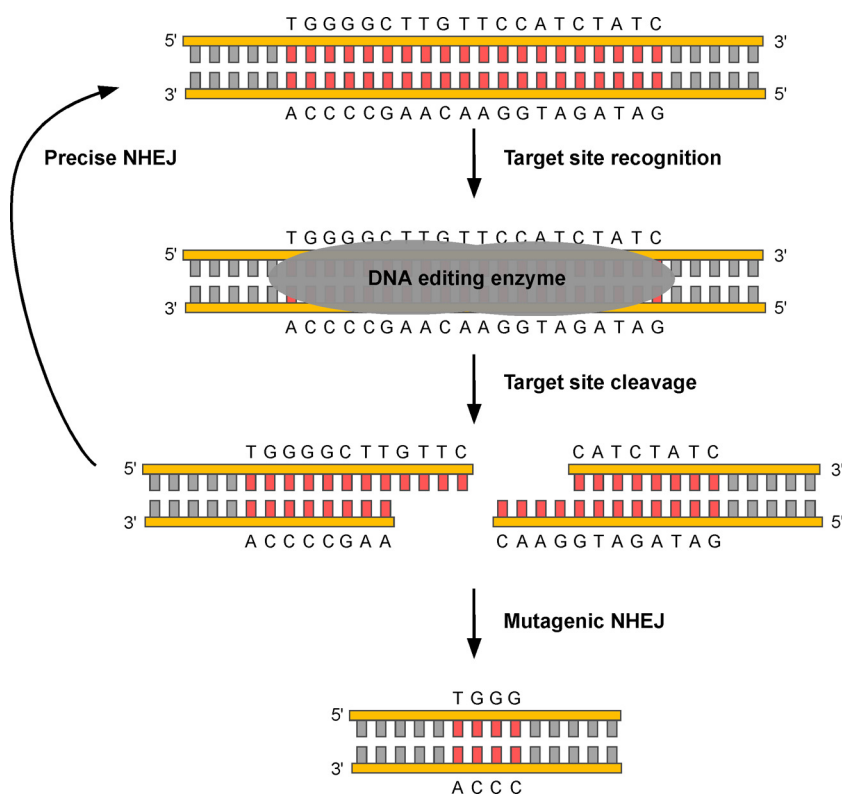


FIG 3 Targeted gene knockout by DNA-editing enzymes. The target sequence (red) is bound by the enzyme, leading to a DNA double-strand break. HE cleavage leaves 3' overhangs as depicted here; ZFNs and TALENs leave 5' overhangs. Such DNA double-strand breaks are typically repaired by precise nonhomologous end joining (NHEJ), which restores the original sequence. However, in the continued presence of DNA-editing enzymes, repeated rounds of cleavage and repair ultimately lead to mutagenic NHEJ pathways. In the example shown here, there is a deletion of 16 bp, resulting in a loss of amino acids and a frameshift mutation in the encoded protein. Small deletions such as this are common, although larger deletions and more-complex insertions/deletions can also be observed.

functional protein, making this an attractive strategy for genetic therapy.

As noted above, targeted DNA endonucleases have already been applied successfully for the knockout of the HIV coreceptor CCR5 (75, 137). We believe that these same endonucleases might also be used to directly target latent or persistent viral genomes, conceptually offering the possibility of a cure for otherwise life-long infections (8, 39). We will consider the differing biologies of three such infections (HIV, HBV, and HSV) and the implications that their biology has on the potential for curative therapy using targeted DNA endonucleases.

GENERAL CONSIDERATIONS IN THE USE OF DNA-TARGETING ENZYMES FOR VIRAL INFECTIONS

If DNA cleavage enzymes can successfully enter infected cells and eliminate production of functional viral proteins, then a critical remaining challenge will be to determine whether these enzymes can be vectorized and incorporated within therapeutic regimens that can completely eliminate large reservoirs of latently infected cells. The challenge of repeatedly delivering nucleases to target cells cannot be overstated. The discovery process will need to include cell culture experiments to obtain quantitative measures of latent viral inactivation, followed by dose escalation studies in animal models of infection to test for efficacy. As with antiviral therapy, dynamical mathematical modeling of enzyme delivery and latent pool kinetics will be essential at each step (135) to precisely define barriers to eradication. Potential hurdles may include

inadequate enzyme delivery to infected cells located within anatomically sequestered sites and reexpansion of the pool of latently infected cells between cleavage enzyme doses.

Pharmacokinetic (PK) and pharmacodynamic (PD) properties of DNA cleavage enzymes are conceptually different from those of antiviral medications. Unlike antiviral agents, which exert an effect only when present at adequate concentrations, cleavage enzymes will have a permanent effect on latent genomes within infected cells. On the other hand, cells with inactivated latent viruses that have been cleaved may quickly become susceptible to reinfection. Therefore, residual viremia in the case of HIV or HBV or persistent viral reactivations in the case of HSV may rapidly reseed the latent pool of cells. Suppressive antiviral therapy may be necessary prior to and during therapy with cleavage enzymes. In addition, repeated cleavage enzyme dosing at narrow time intervals may be needed to prevent reaccumulation of the latent pool of infected cells.

As with antiviral therapy, drug resistance to specific enzymes is a potential problem. While in some cells, an enzyme may fail to cut all the available recognition sites, a more worrisome situation would occur if a genetic mutation destroys the enzyme recognition site but induces a deletion of only 3 or 6 nucleotides, resulting in the loss of 1 or 2 nonessential amino acids. In certain circumstances, this event might leave a functional viral protein and a latently infected cell which is immune to any further activity from the same enzyme. A similar scenario can be imagined for a virus

TABLE 1 Considerations for cleavage enzyme therapy for HIV, HBV, and HSV infections

Consideration	Information for infection with:		
	HIV	HBV	HSV
Potential target sites			
Genome size (kb)	9.7	3.2	154
No. of transcriptional ORFs	9	4	>74
No. of essential viral proteins or transcriptional ORFs	11 essential viral proteins	4 essential transcriptional ORFs	31 essential transcriptional ORFs
Target site mutability			
Mutability	High	Low to intermediate	Low
Polymerase mutation rate	2×10^{-5} mutations/site/cycle	1.4×10^{-5} - 3.2×10^{-5} mutations/site/year	3×10^{-8} mutations/site/cycle
Size/composition of latent reservoir	10^7 latently infected cells; diverse, poorly characterized reservoir	>99% hepatocytes; $\sim 2 \times 10^{11}$ hepatocytes; evidence of diffuse infection in multiple organs and PBMCs (unclear significance)	2-10% of ganglionic neurons (HSV-1); $\leq 20,000$ neurons; presumably only neuronal tissue
Burden of infection per cell	PBMCs, 1 genome/cell; spleen, ≤ 5 genomes/cell	1-50 genomic copies/hepatocyte	2-50 genomic copies/neuron
Maintenance of latency	Homeostatic proliferation of latent pool and/or reseeding due to low-level ongoing replication	High burden of infection in liver due to nonlytic viral replication; turnover of hepatocytes with cccDNA	Permanent infection of neurons during primary infection vs reseeding of neurons
Localization of latent genome	Proviral HIV-1 DNA incorporated into host genome	Extrachromosomal episomal cccDNA	Extrachromosomal episomal viral DNA forms
Key relevant molecular features of latency	Steric considerations due to repressive chromatin at the LTR, including loss of activating histone modifications, presence of repressive modifications, and presence of DNA methylation	Steric considerations due to chromatin surrounding the cccDNA minichromosome	Posttranslational histone modification of LAT; terminally differentiated neurons

that gains a mutation within the enzyme recognition sequence that prevents binding or cleavage without affecting viral fitness. Such escape is analogous to the development of HIV resistance to antiviral agents. In clinical practice, drug resistance to HIV-1, hepatitis B, and hepatitis C is minimized by concurrent use of multiple agents. A comparable approach will probably be necessary for cleavage enzyme-based curative therapy.

One of the most important challenges to the use of DNA cleavage enzymes in treating viral infections is the method of delivery used to target an enzyme to the site of latent infection. As with all genetics-based therapies, the target cell types for latent viral infections vary from one virus to another. Latently infected cells exhibit little to no viral gene expression, and vectors will need to bind and enter a given cell type whether it is infected or uninfected. While successful gene therapy has been established with adeno-associated virus (AAV) and retroviral vectors for human metabolic disorders (3, 119), it is unknown whether certain sequestered tissues, such as the nervous system, may serve as sanctuaries from successful delivery. Furthermore, the target cell types can even vary between patients with the same infection. For example, a latent HSV infection in one patient might reside in the dorsal root ganglia, while in another, it might reside in trigeminal ganglia (TG). While these latent reservoirs are in similar cell types, the challenges of enzyme delivery to each site are different and make the method of delivery important.

A number of factors should be taken into consideration when choosing which of the many available gene delivery vehicles would best suit a given therapy. Is there a nonviral or viral gene delivery system that can efficiently target the cell types harboring latent infection? Does the chosen delivery system need to be adminis-

tered locally, or can a systemic administration route be used? Is the chosen gene delivery system toxic to either the target cells or other organs and cell types exposed upon administration? Will the therapy require transient or persistent expression of the DNA-editing enzyme within the target cell population? Will delivery of high levels of the DNA-editing enzyme result in target cell toxicity? All of these factors should be considered individually before choosing which gene transfer vector suits a given therapy best.

HUMAN IMMUNODEFICIENCY VIRUS

Anatomic sites of HIV-1 latency for cleavage enzyme delivery. While HIV represents the most urgent target for DNA cleavage enzymes, the nature of HIV-1 latency poses tremendous challenges (Table 1). Latency is formally defined as a state of reversibly nonproductive infection of individual cells (170). A subtype of cells harboring latent genomes is termed a reservoir. A phylogenetic definition of reservoirs which contain latent viruses has been established: because reservoirs are continually seeded during active viral replication and because HIV-1 mutates considerably during the lifetime of the human host, reservoirs harbor considerable genetic diversity, though on average, strains diverge less dramatically from a common ancestor than do nonreservoir strains. This occurs because reservoirs include sequences from throughout the phylogenetic tree, including those archived soon after primary infection (122). A compartment is a site, which may be defined anatomically, in which independent evolution, which is substantially divergent from the common ancestor, continues to occur. While both compartments and reservoirs may also serve as sanctuaries where drug delivery is poor (123), the reservoir is not targeted by current antiviral therapies and, as such, is the critical

target for endonuclease therapies. A major current research goal is to define reservoir sites and characteristics.

The most important HIV-1 reservoir consists of memory CD4⁺ T cells, as these cells harbor integrated HIV DNA but do not permit viral transcription (31, 213). Presumably, these cells are formed when a small subset of actively infected cells naturally reverts to a memory state. Replication may resume within a memory cell when the cell transforms back to an effector state upon contact with a cognate antigen or cytokine (14, 57, 116). However, many memory cells never leave a resting state and never express host transcription factors that stimulate viral reactivation, thereby explaining lifelong persistence of latency.

Other possible anatomic and cellular reservoirs of HIV-1 latency in the human host remain relatively uncharacterized (172). Studies of suppressive HIV-1 therapy reveal at least three phases of viral decline (134). The second stage of viral decay initiates after rapid first-phase decay and is considerably slower than primary decay, irrespective of the antiviral agent (66). While primary decay represents death of actively infected CD4⁺ T cells, the source of second-phase decay remains unknown (172). Third phase is characterized by very-low-level residual viremia and appears to last for the lifetime of the infected host. Residual viremia persists with treatment intensification and therefore is likely to represent the release of integrated virus from stable reservoirs of CD4⁺ memory T cells rather than ongoing replication (58). Sequence analysis of residual free virus in the plasma of patients undergoing HAART compared to provirus in resting CD4⁺ T cells or lymph nodes indicates that virus detected in plasma overlaps somewhat with the latent CD4⁺ T-cell reservoir but may also arise from another, as-yet-unidentified cellular source (9, 16). Proposed alternative reservoirs include dendritic cells, macrophages, astrocytes, and hematopoietic stem cells (22, 23, 83, 89, 194), though none of these sites have been firmly established to harbor replication-competent integrated HIV DNA for prolonged durations of time.

Latent reservoir heterogeneity may be of high importance when considering curative regimens: if cleavage enzymes are delivered to a high proportion of one latent reservoir but poorly access another, then this may impact the feasibility of eradication or, at a minimum, the number of doses needed to achieve eradication. The possibility of anatomically sequestered compartments, which also serve as viral reservoirs, must therefore be considered. It is postulated that gut-associated lymphoid tissues (GALT) (111), the central nervous system (CNS) (89), lungs (194), and genital tissues (21) may be sites of latency. Animal models of HIV latency in nonhuman primates under HAART have recapitulated many of the findings in infected humans, including early infection of the CNS (44). If these sites also serve as sanctuaries from vector delivery, then they may prohibit eradication and allow viral replication when ART is eventually stopped.

HIV-1 genome and cleavage enzyme targeting sites. The HIV-1 genome is a challenging target due to its small size of ~9.7 kb. However, the genome is gene-rich, containing nine open reading frames (ORFs) that produce 15 proteins, 11 of which are essential for viral replication. Furthermore, the final gene products from the *gag*, *pol*, and *env* genes are produced via proteolytic cleavage of a polyprotein (128), which means that a deletion or frameshift upstream may disrupt all downstream proteins within the polyprotein. Thus, mutation of a single target could knock out the function of several proteins at once.

Another issue that may hinder efforts to cure HIV-1 is the

extremely high mutability that the virus exhibits during replication, presumably as a means of immune evasion. During the HIV infectious cycle, the viral RNA genome is reverse transcribed by the virally encoded reverse transcriptase (RT) to produce the DNA provirus. Reverse transcription has a high error rate of 1.4×10^{-5} to 4×10^{-5} mutations/base pair/replication cycle (1, 105), resulting in a virus population that is genetically diverse with a complex fitness and mutational landscape. Mathematical models predict that within a single HIV-infected patient, every possible nucleotide substitution is represented (202). Despite the fact that cleavage enzymes seek to eliminate virus from reservoirs in which viral replication is rare or absent altogether during antiretroviral therapy, nucleotide sequence diversity is likely to be high due to continual seeding of the reservoir throughout infection as the virus evolves in the context of immune pressure (122). Thus, use of a single DNA-editing enzyme may select for resistant viral variants, and successful therapy will likely require simultaneous use of multiple enzymes targeting different regions of the virus, as discussed in more detail below. On the other hand, the high mutation rate of HIV-1 comes at a significant fitness cost: many mutations render viral particles incapable of further infection and replication (73). Therefore, not all nucleotide changes at cleavage enzyme target sites will prove deleterious toward eradication efforts.

Latent HIV-1 DNA viral load. Despite the challenges of reservoir diversity and genome mutability, the number of cells harboring integrated HIV DNA is estimated to be quite low (~ 10^7 infected cells) (29), a fact which may increase the feasibility of a cure. Recent studies also suggest that most latently infected peripheral blood mononuclear cells (PBMCs) contain only one integrated HIV-1 provirus (80), again consistent with an overall low burden of infection. Yet PBMCs may not be representative of the most-critical anatomic regions of latency: in one study, infected splenocytes harbored a median of 5 proviral copies (81). Moreover, it is unknown whether the size of the latent reservoir varies considerably between infected persons and whether such variability impacts disease phenotype.

The establishment of a latent reservoir occurs with systemic spread of virus early during primary infection. Even patients who initiate HAART during the first weeks after viral acquisition are rendered incurable of persistent infection with currently available interventions (32, 174). Macaque challenge studies suggest that viral replication successfully bypasses mucosal immune control and disseminates widely throughout lymphatic tissue in the gut within 2 weeks after simian immunodeficiency virus (SIV) inoculation into the genital tract (192). Depletion of gastrointestinal CD4⁺ T cells is likely to coincide with establishment of reservoirs of latent infection, though within infected tissues, the proportion of target cells that are directly infected is small (29). If ART is not administered immediately after a high-risk exposure to HIV and infection takes hold, then the latent reservoir is irreversibly established within 10 days of primary infection (30).

During the 8 to 10 years of untreated infection that typically precede AIDS and death, plasma CD4 counts slowly decrease while the latent reservoir diversifies and slowly diverges from the founder strain. In patients who receive HAART, latently infected cells survive for decades despite the absence of high-level viremia and the immune cell depletion. Even successfully treated patients, who have no detectable HIV DNA in their peripheral CD4⁺ T cells or GALT and appear to have complete elimination of viral replication, experience a rebound of viremia within 50 days of cessa-

tion of therapy (32). This indicates that an undetectably small CD4⁺ T-cell or other reservoir that is either persistent or constantly replenishing is able to reconstitute infection (33) and will also have to be targeted with cleavage enzyme therapy.

The long-term mechanism that maintains the latent infection despite HAART is of importance when considering viral eradication strategies. One possibility is that resting CD4⁺ T cells are reseeded with low-level replication within the activated CD4⁺ T-cell compartments (33, 146). Proviral HIV-1 replication can be induced in latently infected CD4⁺ T cells (34). One study suggests that high multiplicity of infection within single cells renders HIV resistant to complete elimination (169), though this phenomenon has not been observed *in vivo*. This model of viral persistence assumes the presence of anatomic drug sanctuaries as a mechanism to support ongoing viral replication.

However, there are several findings to suggest that viral replication is nearly completely eliminated during successful HAART. Development of *de novo* drug resistance clones does not occur in circulating virions during fully suppressive HAART (84, 206). Moreover, viral blips persist at an unchanged rate following ultra-intensification of regimens with four or more agents (48), and it is now believed that current ART regimens bind viral replication enzymes cooperatively and potentially suppress nearly all replication (164, 165). Finally, recent mathematical models suggest that the latent pool of infected cells is not replenished due to low-level viral replication despite HAART and that the stability of the reservoir can be attributed to the long life span and occasional activation of latently infected cells (153, 161). The HIV reservoir includes T cells from two distinct populations: central memory T cells (T_{CM}) and transitional memory cells (T_{TM}). The long life of the T_{CM} and the low levels of antigen-driven proliferation lead to the stability of this population, although it is slowly depleted over time (27). On the other hand, it has also been proposed that latently infected T_{TM} persist via interleukin-7 (IL-7)-mediated homeostatic proliferation, which preserves not only the size of the reservoir but also its genetic variability. HIV-1-infected hematopoietic stem cells (HSCs) have been recently reported *in vivo* (22, 23), and these cells, with their capacity to self-renew over the lifetime of the individual, may possibly represent another component of the HIV reservoir. Yet more-recent reports identify no HIV DNA within CD34⁺ bone marrow progenitor cells (52, 79).

Molecular features of HIV-1 latency. During latency, the integrated HIV provirus is transcriptionally repressed by a number of mechanisms which may impact success of the cleavage enzyme activity. When the provirus integrates into actively transcribed host genes, which occurs >90% of the time, transcriptional read-through from the upstream promoter can prevent formation of the initiation complex at the long terminal repeat (LTR) (72, 95, 160). In addition, resting cells lack the levels of host transcription factors necessary for robust viral gene expression (116, 181). The formation of repressive chromatin at the LTR—including loss of activating histone modifications, presence of repressive modifications, and presence of DNA methylation—has also been implicated in HIV latency (82, 132, 191). The accessibility of the DNA to recognition and cleavage by therapeutic enzymes may be compromised by the presence of heterochromatin and possibly by nucleosome positioning.

Gene delivery and genome-targeting efforts to date for HIV-1. One novel approach to the problem of HIV latency is the use of gene editing to render T cells resistant to HIV. Initial exper-

iments in mice have used a variety of approaches to downregulate CCR5, an entry receptor for HIV-1 strains (5, 47, 86, 91, 99, 166). Cells deficient for CCR5 expression would theoretically expand and become enriched due to the selective advantage conferred by resistance to HIV infection. If this therapy succeeds, then latent reservoirs would be unable to propagate widespread infection. This approach holds promise based on an apparently successful cure of HIV-1 achieved in a leukemia patient who was transplanted with CCR5Δ32/Δ32 stem cells (4, 78), although chemotherapy, radiation, antithymocyte globulin, and graft-versus-host disease may have all contributed to HIV eradication in this case.

Accordingly, there have been promising attempts to permanently knock down or knock out CCR5 expression with the use of engineered Zn finger nucleases (ZFN) that permanently disrupt the CCR5 gene in cells. Fifty percent of CCR5 alleles were disrupted using this technique: when treated human CD4⁺ cells were transplanted into a humanized mouse model, the mice exhibited lower viremia and slower decay of CD4⁺ cells after infection with HIV compared to mice transplanted with untreated cells (137). The same approach was used to decrease CCR5 expression in human hematopoietic stem cells (HSCs), although at a much lower frequency than in terminally differentiated cells: treated HSCs developed into multiple lineages of progeny in which CCR5 was nonfunctional. These engineered cells also successfully engrafted in a humanized mouse model, again allowing for lower viremia and less-severe CD4⁺ depletion after HIV-1 challenge (75).

Based on these encouraging results, Zn finger nucleases that cleave CCR5 have entered phase I clinical trials. Adverse effects due to the loss of CCR5 function are a concern. CCR5Δ32 has been implicated in more-severe disease during West Nile virus infection (61, 102), indicating an important role in certain protective immune pathways. In addition, all therapies targeting CCR5 can potentially select for the more pathogenic CXCR4-tropic HIV-1. However, ZFNs have also been used to target CXCR4 (204), which is not essential for normal T-cell function.

A second approach would modify the provirus within latent cells, thus preventing reactivation. This technique would not require adoptive transfer but would have challenges associated with delivery. One of the first attempts to target the latent provirus directly involved the use of a site-specific recombinase (SSR) to selectively excise integrated provirus. Recombinant HIV containing *loxP* sites in the U3 region of the long terminal repeat (LTR) was excised after integration by Cre recombinase (56). A Cre-derived SSR that recognized the HIV LTR successfully excised an integrated provirus containing the targeted LTR (157). However, the LTR sequence was chosen based on its similarity to the Cre recognition site and was atypical for HIV. LTR sequences from other HIV strains are much more divergent from the wild-type Cre recognition sequence. Directed evolution of these recombinases has been shown to change their sequence specificity, so it is possible that SSRs that recognize a variety of HIV LTRs may be generated (15, 19, 20).

Our lab recently published a more general demonstration of the ability of homing endonucleases to target and inactivate integrated virus (8) without excision of proviral DNA. We developed a lentivirus reporter bearing a recognition site for the HE Y2-*AniI*, a variant of I-*AniI* (178), inserted between the translational start site and the coding sequence for a short-half-life green fluorescent protein (GFP) reporter. Cleavage of the target site and subsequent repair by nonhomologous end joining caused small insertions and

deletions at the site of cleavage, resulting in frame shifts, loss of the translational start site, or disruption of essential GFP-coding sequences. Thus, successful HE attack was monitored by loss of GFP expression. We found that Y2-*AniI* could efficiently target the integrated reporter lentivirus, resulting in mutagenesis of the target region in over 97% of Y2-*AniI*-expressing cells without evidence of detectable toxicity. These data suggest the possibility that incorporated proviral DNA can be selectively disabled without excision and without damage to the host cell.

As mentioned above, there are two potential strategies for a genome-targeting-based approach to HIV treatment. Strategies aimed at both the integrated provirus and cellular genes that permit HIV infection are promising but require enzyme delivery to specific but distinct cell types. For an approach targeting integrated HIV provirus, targeting enzymes to CD4⁺ cells showing active HIV replication in addition to CD4⁺ memory T cells that harbor reservoirs of latent HIV infection would be desirable. In contrast, the disruption of cellular genes that permit HIV infection would ideally be done in CD4⁻ HSCs that would, if unaltered, give rise to CD4⁺ T cells permissive for HIV-1 infection (85). Although the disruption of cellular genes in CD4⁺ T cells can prevent HIV spread (137), HSC disruption would prevent reseeding of CD4⁺ T-cell reservoirs following therapy.

Efficient gene delivery to cells of hematopoietic origin, including CD4⁺ and CD34⁺ cells, can be achieved using a number of different approaches, and the methods available to investigators have been reviewed in detail elsewhere (117). In the context of DNA-editing enzyme delivery, the number of studies in CD4⁺ and CD34⁺ cells is small, although efficient enzyme delivery and genome targeting have been achieved. The nonviral Amara nucleofection gene delivery system has been used to efficiently deliver ZFNs targeting the IL-2R gamma gene or CCR5 to CD4⁺ T cells and CD34⁺ HSCs in *ex vivo* gene transfer protocols (189). Targeted CD34⁺ cells were able to engraft upon transplantation into immunodeficient mice, demonstrating the efficacy of this approach. Other groups have chosen viral vectors to target CD4⁺ and CD34⁺ cells. Adenovirus vectors that use CD46 as an entry receptor have been used to efficiently deliver ZFNs to CD4⁺ T cells that could engraft upon transplantation into immunodeficient mice (137, 204). Nonintegrating lentivirus vectors (also referred to as integrase-deficient lentiviruses [IDLVs]) have also successfully been used to deliver gene-targeted ZFNs to CD34⁺ cells (104). Although only a few delivery systems have been used to deliver DNA-editing enzymes to CD4⁺ and CD34⁺ cells, the existing data suggest that genome targeting could be a successful strategy for the elimination of HIV infection. However, delivery to every single infected cell may ultimately be necessary to achieve a cure.

HEPATITIS B VIRUS

Anatomic sites of chronic HBV infection for cleavage enzyme delivery. HBV is primarily an infection of the liver, an organ that is highly vascularized and receives approximately 25% of systemic blood flow, with 33% and 67% contributions from arterial and portal circulation, respectively. Within the liver, specialized, fenestrated capillaries, called sinusoids, allow hepatocytes to be continually bathed in blood. Moreover, enzyme entry into hepatocytes occurs prior to first-pass hepatic metabolism. For these reasons, cleavage enzyme delivery to the liver should occur at high levels.

A potential challenge is that HBV infection disseminates widely beyond the liver: various investigators have detected viral DNA and surface antigen in lymph nodes, spleen, bone marrow, kidney, skin, gastrointestinal tract, pancreas, testes, and periaxonal ganglia. A broad range of cell lines, including endothelial cells, epithelial cells, neurons, macrophages, peripheral blood mononuclear cells, and polymorphic nuclear leukocytes are permissive for HBV replication, and extrahepatic manifestations of disease include medium- and small-vessel vasculitis, glomerulonephritis, aplastic anemia, myocarditis, and polyarthritides (45, 107, 139, 142, 205, 211). It is unknown whether these extrahepatic sites could serve as viable reservoirs for reseeding of the liver following isolated eradication of cccDNA genomes from liver cells.

HBV genome and cleavage enzyme target sites. Several features make HBV an attractive candidate for eradication with cleavage enzymes (Table 1). Though HBV has a very small genome (3.2 kb), viral survival is highly dependent on a small number of viral proteins for replication. The HBV genome comprises four open reading frames (envelope, nucleocapsid, polymerase, and X protein) which are translated into only seven proteins. The two nucleocapsid products are hepatitis B virus c and e antigens (HBcAg and HBeAg, respectively). HBcAg is involved in viral packaging, while HBeAg plays a possible role in immunosuppression (112). A cellular immune response to these antigens is thought to be important for viral clearance (133). The envelope comprises three polypeptides, designated the large, medium, and small surface antigens, which are all heavily glycosylated (18). The X protein, which is absolutely essential for viral replication, can modulate host and viral gene expression as well as affect host-cell signal transduction (217). Specifically targeting any one of these proteins would likely be sufficient to greatly reduce or eliminate viral replication.

The polymerase protein includes reverse transcriptase, DNA polymerase, and RNase H domains and is essential for encapsidation and replication of the viral genome through the reverse transcription process. After arriving in the nucleus, the HBV genome is converted from a partially double-stranded relaxed closed DNA (rcDNA) into a covalently closed circular form (cccDNA) (121). cccDNA is the template for all viral protein synthesis and viral replication through a DNA-to-RNA-to-DNA mechanism that requires viral reverse transcriptase. Integration of the genome is not required for replication (118), and cccDNA exists in an episomal state.

Two mechanisms likely contribute to cccDNA persistence and are of interest for eradication strategies via cleavage enzymes. First, the cccDNA pool is expanded and maintained by a mechanism in which newly produced rcDNA, whose principle destination is in nascent budding viral particles, instead is recycled back into the nucleus and converted into additional cccDNA copies (88, 184). In addition, hepatocyte division can result in asymmetric distribution of HBV in progeny cells, which can be one way that HBV is eliminated (212). The kinetics of these two processes will impact the number and frequency of doses required for eradication of cccDNA stores.

The HBV reverse transcriptase enzyme induces mutations at a considerably lower rate (1.4×10^{-5} to 3.2×10^{-5} mutations/base pair/year) than HIV (2×10^{-5} mutations/base pair/day) (103). It is estimated that in a typical patient during a 24-hour period, 10^{12} HBV particles are produced and cleared: based on the mutation rate and genome length, 9×10^{10} and 4.5×10^9 single- and dou-

ble-base pair mutants, respectively, are also produced, thus exceeding the total possible numbers of single- and double-base pair mutants within the HBV genome by factors of 10 million and 100, respectively (136). As a consequence, current antiviral therapies can cause resistance mutations, leading to a poorer overall prognosis. A successful strategy for eliminating cccDNA from hepatocytes may need to account for an abundance of diverse circulating particles. To this end, cleavage enzymes may not be effective if suppressive antiviral therapy is not initiated first to lower the burden of replicating and mutating HBV.

On the other hand, there are several lines of evidence suggesting that cccDNA may exhibit less sequence diversity within a host. First, HBV is intolerant to many mutations. Drug-resistant HBV tends to emerge months to years after initiating treatment rather than immediately, as with hepatitis C and HIV-1 treatment failure (152, 180). This suggests that HBV resistance is not the primary cause of viral escape from antiviral therapy (42). Second, HBV reverse transcriptase can remove newly incorporated nucleotides during replication, allowing for ongoing proofreading of the HBV genome (188). Moreover, work in a duck model of infection suggests that superinfection of preinfected hepatocytes with a second drug-resistant strain cannot occur, even if the second virus possesses a replicative advantage (197).

The HBV genome is also not exceedingly diverse globally: there are 8 known HBV genotypes, each of which has less than 4% within-clade sequence divergence, while divergence exceeding 8% and sometimes approaching 16% can be observed between different clades (141). HBV therefore appears to survive within a fairly limited evolutionary space. Importantly, several regions, including the spacer region of the polymerase ORF, are conserved across all genotypes (Z. Chen, personal communication). Successful targeting and cleavage of a sequence within this region could feasibly cause mutations and/or frameshift insertions/deletions that render the polymerase gene product nonfunctional. It will be crucial to target such highly conserved target sites. Sequence diversity within potential target sites may also be overcome by targeting multiple sites concurrently.

Latent HBV viral load. A major hurdle to eradicating HBV will be the massive pool of infected cells compared to those of HIV-1 and HSV. Due to the nonlytic nature of HBV replication, infected cells turn over only slightly more rapidly than uninfected cells according to the intensity of the host T-cell response. HBV therefore has sufficient time to spread efficiently between hepatocytes. The use of PCR *in situ* hybridization technology has revealed that in persons with chronic active hepatitis B and wide ranges of plasma viral loads ranging from 10^3 to 10^9 HBV DNA copies/ml, virtually all hepatocytes harbor HBV DNA. On the other hand, HBV RNA and surface antigen, which are markers of replication, are present only in certain hepatic regions (127). HBV DNA was also detected in a mean of 5% of hepatocytes in persons with occult hepatitis B (positive low-level plasma HBV DNA with negative HBV surface antigen); moreover, 50% of subjects had detectable levels of HBV in circulating PBMCs (150). Hence, during all forms of chronic infection, HBV is widely disseminated throughout the liver, and in active disease, nearly all of the total 2×10^{11} hepatocytes are infected.

If uninfected hepatocytes emerge during infection as a result of asymmetric cccDNA homeostatic proliferation (212), these cells are presumably at high risk of rapid infection due to high levels of surrounding HBV virions. Mathematical models suggest that un-

der certain circumstances, regenerating hepatocytes may be refractory to infection based on the surrounding cytokine milieu: this is a key feature of the proposed mechanism for viral clearance, which occurs in approximately 90% of acutely infected adults (35). It is estimated from animal models that the entire supply of hepatocytes turns over 1 to 3 times during the first year following acute infection (67, 115, 175). Similar rates of infected cell turnover are probable during chronic active infection. Given that cleavage enzyme therapies are unlikely to enhance host immunological responses, replenished cells will likely remain largely susceptible to HBV infection, potentially adding kindling to the fire. The regeneration of target cells will present a challenge to curative strategies and may necessitate prolonged courses of therapy.

Treatment strategies that attempt to target the entire pool of hepatocytes containing viral genomes will need to account for the total genomic burden of infection rather than just the number of infected cells. Fortunately, the genome persists at relatively low levels within each infected cell. Individual nuclei from duck liver cells infected with duck HBV were isolated and analyzed with nested PCR: a significant fraction of all nuclei (13%) contained exactly one copy of cccDNA, and few nuclei contained more than 50 cccDNA copies (212). These data correlate with viral load measurements from human livers that were standardized to 10^6 total hepatocytes: in chronically infected and untreated patients, the total genomic burden of cccDNA exceeded the number of infected cells by only a maximum of one log. On the other hand, cccDNA represented only 0.7 to 22.0% (median, 5.9%) of total HBV DNA within the liver, highlighting the need for concurrent use of antiviral therapy with cleavage enzyme therapies (93).

The quantity of total liver cccDNA varies enormously between chronically infected persons (93), likely as a function of the strength of the immunological response. Indeed, a patient's outcome after acute HBV infection appears to hinge on their immune status rather than a specific latency-inducing mechanism contained in the virus. Viral expansion during acute infection is limited via noncytolytic reduction of viral proteins by cytokines produced by activated T lymphocytes, while clearance of plasma viremia correlates with an intense cytolytic T-cell response (59, 65, 148). Accordingly, chronic infection results from an inadequate initial lymphocyte response to viral antigens (130, 148), though other components of the immune response may be lacking as well (64). The ability to contain infection is age dependent: 1 to 5% of adults and 90% of neonates infected with HBV fail to develop a sufficient immune response to clear the virus and develop chronic, persistent infection (207).

Despite the importance of immunological control in determining clinical outcomes, nonreplicating viral forms partially avoid cytolytic effects. Even after apparent clinical clearance of viral surface antigen from plasma and conversion to a positive antibody status during occult infection, a high burden of cccDNA remains within infected hepatocytes (150). HBV e antigen (HBeAg) is commonly used as a marker of viral replication and infectivity. In untreated chronic HBV patients positive for HBeAg, cccDNA copy numbers are higher than they are in untreated HBeAg-negative patients (3.0 median copies/cell and 0.31 median copies/cell, respectively) (93). Despite this lower viral genome load, intrahepatic cccDNA maintains replicative capacity even in asymptomatic, HBeAg-negative carriers. This accounts for the high probability of viral reactivation in severely immunocompromised hosts who have definitive evidence of previous clearance of

virus (71). In addition, asymptomatic carriers who are positive for HBsAg and negative for HBeAg and who maintain normal liver function yet are unable to clear the virus through adequate immune system activation are at risk for developing hepatocellular carcinoma, indicating incomplete viral control (210).

cccDNA remains in the nucleus for the lifetime of the infected hepatocyte, which may span months to years (2), and is the probable source for partial viral rebound that occurs in approximately 34% of patients during antiviral therapy, even in the absence of drug resistance (76). Accordingly, although total clearance of HBV DNA from plasma occurs 90% of the time with optimal treatment regimens (25, 92), this does not correlate with cccDNA eradication and relapse is common after therapy is stopped, particularly in the eAg-negative disease state (168). Successfully treated patients achieve only a median 0.8-log reduction in cccDNA levels compared to untreated patients (203). Moreover, their decrease in cccDNA levels does not coincide with a reduction in the percentage of hepatocytes displaying markers of HBV infection.

Molecular features of HBV latency. cccDNA exists as a minichromosome in the form of a nucleosome similar to host cell chromatin (121). Minichromosome structure has important regulatory effects on the transcription of viral genes and may also influence the accessibility of target sequences to DNA-recognizing enzymes.

Gene delivery and HBV genome-targeting efforts to date. There are few examples of gene therapy approaches targeting HBV. One possible explanation is the lack of an adequate cell culture model for HBV infection (43). RNA interference (RNAi)-based approaches have been investigated (43, 167) but are limited because they do not directly eliminate episomal viral cccDNA and, rather, only slightly reduce the cccDNA pool via an effect on the recycling of viral DNA back into the nucleus (187). Zinc finger proteins (ZFPs) and zinc finger nucleases (ZFNs) have been used to target viral DNA. A specifically designed ZFP avidly bound to duck hepatitis B virus DNA after delivery to the nucleus via attachment to an SV40 nuclear localization signal. While ZFPs do not cleave DNA due to their lack of endonuclease activity, this approach resulted in substantial reductions in viral RNA, proteins, and viral progeny in cell culture (215). ZFNs can cleave both episomal and proviral DNA, followed by repair through highly error-prone nonhomologous end joining. In cell culture experiments, an HBV-specific ZFN showed site-specific cleavage leading to a reduction in pregenomic RNA levels without a loss in cell viability (39).

As the liver is readily accessible to a number of gene delivery vectors, any novel anti-HBV therapy has an inherent advantage, since almost the entire target cell population can be treated. Furthermore, several vectors that efficiently transduce the liver are readily available, have been well characterized, and have even been used in clinical trials. The most-common gene delivery systems for hepatic gene transfer are recombinant adenovirus (Ad) and adeno-associated virus (AAV) vectors which can efficiently transduce the liver without the need for an invasive delivery procedure. Although other methods of hepatic gene transfer are available (74), the high efficiency of gene transfer seen with Ad and AAV vectors makes them ideal candidates for the delivery of anti-HBV therapeutics. Upon intravenous delivery of a relatively moderate dose, Ad vectors can transduce 100% of hepatocytes with minimal toxicity (96). Consequently, Ad vectors have been used to deliver

anti-HBV RNAi sequences to the livers of HBV transgenic mice with considerable success (40, 147). AAV vectors are also able to efficiently transduce the liver after delivery of moderate vector doses that cause minimal toxicity (216). They have also been used to deliver anti-HBV RNAi sequences to the livers of HBV transgenic mice with significant success (26, 60).

HERPES SIMPLEX VIRUS

Anatomic sites of HSV latency for cleavage enzyme delivery. The major issue unique to HSV latency is that it occurs exclusively in anatomic sanctuaries (the trigeminal ganglia, dorsal root, and spinal cord) (129) (Table 1). HSV establishes latency during primary infection. After replicating in epidermal cells in oral (HSV-1) or genital (HSV-2) skin and mucosae, HSV invades the peripheral nervous system and spreads from neuronal endings via axons to cell bodies in the ganglia (151). HSV infection in epidermal cells is rapid and lytic, resulting in characteristic oral (usually HSV-1) and genital (usually HSV-2) ulcers with dense inflammatory infiltrates (41, 214), while infection in neurons leads to limited tissue damage and lytic viral replication only during the first week following infection (69). Recent evidence suggests that while latency may be tightly established at the single-cell level, the ganglia leak virus nearly constantly toward the genital tract (159). Mouse models show early and late transcripts in small numbers of unperturbed ganglionic neurons even in the absence of reactivation (54). Therefore, low-level focal replication is likely to occur even while most neurons within the ganglia remain quiescent.

HSV genome and cleavage enzyme targeting sites. Several features of HSV latency may facilitate the design of cleavage enzymes that target key sites within the latent genome. First, the HSV genome is large and contains numerous essential replication genes. HSV-1 and -2 are enveloped viruses with linear double-stranded DNA genomes that are 152 kb and 154 kb in length, respectively (49, 109). HSV-1 contains approximately 94 transcription units (ORFs), of which 84 encode a protein (145). For HSV-2, 74 ORFs have been confirmed and matched to HSV-1. Out of the 84 HSV-1 proteins with a described function, 31 are essential for replication in culture (151). Introduction of mutations within the DNA reading frame for many viral protein sites may permanently disable HSV genomes, and *in silico* analyses suggest that >100 potentially useful targets may exist (201).

Second, target sites are likely to be relatively well conserved among isolates. HSV DNA genomes are more stable than those of RNA viruses due to an overall low mutation rate (3.5×10^{-8} mutations/site/year) (156) and high accuracy of replication associated with HSV DNA polymerase 3'-to-5' exonuclease proof-reading activity (10). Yet different isolates that have evolved for prolonged periods of time within distinct ecologic niches may vary substantially from one another. The genome sequences of one clinical and one laboratory HSV-1 isolate had 1% sequence divergence compared to the only other sequenced HSV-1 genome (from laboratory strain 17) (177). This level of variability between the three isolates may be an issue for targeted mutagenesis. However, two of the isolates compared were laboratory strains that had artificially undergone multiple passages in culture. In fact, in a study examining the sequence variation of a segment comprising 3.5% of the genome of HSV-2 isolates from different regions of the globe, there was high similarity (99.6%) between the two most distant HSV-2 isolates (125). A phylogenetic analysis of clinical HSV-1 isolates determined the sequence diversity on a region rep-

representing 2.3% of the HSV-1 genome. Depending on the gene of the sequenced region, sequence differences ranging from 0.6% to 3.1% were observed (124). HSV-1 and HSV-2 are closely related at the nucleotide level, with 83% nucleotide identity when HSV-1 laboratory strain 17 was aligned with HSV-2 laboratory strain HG52 (49). Therefore, target sites found in one type have a reasonably high likelihood of being conserved in the other type.

Another concern is that viral escape mutants may emerge and persist within a person over time, as has been observed occasionally with antiviral therapy. Several reports identified that recombinant viruses are shed asymptotically in some infected individuals, suggesting coinfection with more than one virus (101, 179). However, it has not been demonstrated that individual neurons are coinfecting with genetically distinct genomes.

Latent HSV DNA viral load. In nonimmunocompromised individuals, at a single point in time, HSV latency is established in only a small percentage of sensory neurons, ranging from 2.0 to 10.5% for HSV-1 (198). There are approximately 10,000 to 20,000 neuronal cell bodies per ganglion, and approximately 10 ganglia are at risk for infection, though the spinal cord may serve as another reservoir of HSV DNA. The number of HSV genome copies in each latently infected neuron is also modest (2 to 50 copies/cell for HSV-1) in most infected cells (198, 199). This suggests an overall infectious burden of $\sim 10^5$ genomes. In mice, viral burden, defined by copy number and the number of infected cells, appears to be linked to recurrent disease frequency/rate (77). Therefore, it may not be necessary to eliminate all copies of the latent genome within every infected neuron to limit disease severity or completely eliminate viral shedding.

On the other hand, HSV-2 reactivations defined by high-level viral replication in genital skin near sensory nerve endings occur approximately weekly in humans (195). Moreover, human shedding patterns remain mostly unchanged over decades (138), meaning either that a pool of latently infected neurons is permanently established during primary infection or that steady state is achieved via a balance between elimination and replenishment of HSV within neurons. There is indeed some indirect evidence that latency may not be a static process at the cellular level. In human cadaver studies, dense CD8⁺ and CD4⁺ T-cell infiltrates congregate around neurons containing HSV DNA and only some infected neurons produce latency-associated transcripts (193). In mice, CD8⁺ lymphocytes with a cytolytic phenotype inactivate lytic HSV replication, not by killing infected neurons but, rather, by inactivating the immediate early protein ICP4 (87). These data suggest that regional HSV DNA levels in ganglia may possibly fluctuate over time due to local immune pressure. If neurites are seeded with HSV during each mucosal reactivation and the pool of latently infected ganglionic neurons is periodically replenished, then a more comprehensive dosing strategy may be necessary to achieve elimination of latently infected neurons.

Molecular features of HSV latency. If enzyme delivery to an adequate number of infected ganglionic cells is accomplished, then several molecular features of HSV latency are likely to impact whether the latent genome is amenable for targeted mutagenesis. On one hand, latent HSV DNA is extrachromosomal and predominantly maintained in circular or concatenated forms referred to as episomal “endless DNA” (53, 149). This is advantageous because it is perhaps less likely that the DNA breaks and repair of the episomal DNA will lead to cellular chromosomal mutation, as might occur with integrated virus.

However, viral episomes are associated with the nucleosome in a chromatin structure, which may present challenges for endonuclease binding (46). During its latent state, HSV usually does not produce viral progeny and expresses only one transcript, called the latency-associated transcript (LAT). Viral transcription during latency is regulated not by DNA methylation but by posttranslational histone modifications (50, 90). During latency, the LAT region is associated with acetylated histones or active chromatin, whereas lytic-gene promoters are associated with heterochromatin (repressive) forms of histones (90). The presence of acetylated histones may influence how accessible a viral target sequence(s) is to cleavage enzymes. Histones may also present steric impediments to delivery of cellular repair machinery: fortunately, non-homologous end joining is active throughout the cell cycle while homologous recombination (which would not induce lethal mutations in key viral genomic segments) is preferentially active in the S and G₂ phases of the cell cycle (154). Because neurons are likely to be in cellular arrest phase, lethal-mutation-inducing non-homologous end joining is likely to be favored.

Despite being terminally differentiated and postmitotic, ganglionic neurons are highly active cells. The high metabolic and transcriptional activity within neurons increases the potential for genomic DNA damage, necessitating a robust DNA damage response. While all eukaryotic DNA repair systems operate in neurons, DNA repair activity is slower relative to dividing cells and, therefore, genomic errors accumulate more rapidly. Despite the fact that DNA repair is attenuated at the global genome level, it is maintained in expressed genes which are, in turn, less prone to mutation (126). In addition, the homologous-recombination repair pathway may not be essential due to the postmitotic state of the neurons (11). This may also favor nonhomologous-end-joining repair of cleaved viral targets, something which is a prerequisite for effective eradication therapies.

Gene delivery and genome-targeting efforts to date. To date, only one report describes HSV genome targeting by HEs. In cultured nonneuronal cells transiently expressing HSV-specific HEs, 2.8 to 16% of HSV-1 genomes harbored a mutation at the HE target site. In addition, viral replication and infection of cultured cells was substantially diminished when HEs were introduced prior to HSV infection at various multiplicities of infection. This study demonstrated that HSV genomes can be mutated following HE exposure, although cellular toxicity was evident with certain engineered HEs (63).

A few considerations will need to be addressed with all potential delivery systems. While expression of transgenes carried by such vectors has been demonstrated, expression is not sustained over an extended period of time. To resolve this issue, the LAT promoter could be used to drive expression of cleavage enzymes. However, when examined in a cross section, only one-third of infected neurons express LAT, a finding which appears to be due to histone modifications (198). The expression of the transgene under a cytomegalovirus (CMV) promoter or a similar expression promoter may be sustained long enough for genome targeting to occur. An advantage of such a promoter is its self-limiting phenotype, which may limit the potential side effects of long-term expression of homing endonucleases or other cleavage enzymes.

Delivery of the HE to the neurons where HSV is latent could be achieved by using replication-incompetent HSV-based delivery vectors (200), which have been developed for gene therapy of diseases of the nervous system (62). In animal models, HSV-1 and -2

display different tropisms for sensory neurons, with HSV-1 more likely to be detected during latency in neurons displaying the surface marker A5 and HSV-2 more likely to be detected during latency in those displaying KH10 (106). Therefore, the use of HSV-based delivery systems may allow matching of the delivery vectors to the HSV serotype targeted.

Adenovirus vectors have been employed in primary cultures of latently infected trigeminal ganglia (TG) (70). AAV and lentivirus vectors have been considered for gene delivery to dorsal root ganglion neurons and have achieved efficient and sustained transduction within human sensory neurons in dissociated cultures, demonstrating the potential of these vectors for gene therapy applications in the peripheral nervous system (55, 70).

A particular challenge of cleavage enzyme delivery to the ganglia is the blood brain barrier, a structure that isolates and protects the CNS from harmful macromolecules. This is an area of great interest for research fields dedicated to the treatment of brain tumors, Alzheimer's disease, and Parkinson's disease. Various strategies are being investigated to avoid the need for direct intracranial drug delivery. Noninvasive strategies include drug manipulation, carrier-mediated drug delivery, receptor/vector-mediated delivery, and intranasal drug delivery (131, 144), and some of these approaches may prove useful in gene therapy efforts. Nanotechnology applications include the delivery of drugs and other small molecules, such as genes and oligonucleotides, across the barrier (182).

CONCLUSIONS

There is a new focus on strategies that directly target latent viral genomes with the goal of eradicating chronic viral infection. If cleavage enzymes can be successfully targeted to latently infected cells and can subsequently incapacitate key viral proteins, then the possibility of a viral cure exists. Several promising types of cleavage enzymes that target key regions within the HIV-1, HBV, and HSV genomes are in development. When *in vivo* delivery becomes a goal, considerations will need to include the unique pharmacokinetic/pharmacodynamic features of cleavage enzymes, the challenges with specific sites of latency for these infections, the development of viral resistance due to high viral mutability, the density of latently infected cells, and the molecular and steric constraints to enzyme delivery. These challenges call for the concurrent use of cell culture systems, animal models, mathematical models, and strategic clinic trial design in order to maximize the likelihood of success.

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Joshua T. Schiffer is a clinician specializing in infectious diseases with particular interests in the management of HIV-infected patients and other immunocompromised hosts. The aim of his research program is to gain a better understanding of the quantitative features of human pathogens and immune responses. In close collaboration with several colleagues at the Fred Hutchinson Cancer Research Center and the University of Washington, he designs mathematical models that capture growth and decay kinetics of infectious organisms. These models attempt to replicate detailed empirical datasets and, in turn, are used to inform subsequent human studies and laboratory experiments. Model results inform treatment strategies and attempt to identify hypotheses that may ultimately inform certain treatment and prevention strategies.



Martine Aubert obtained her Ph.D in bacterial genetics at the University of Nancy I (France) and did her postdoctoral training on herpesvirus biology with Dr. J. A. Blaho in the Department of Microbiology at Mount Sinai School of Medicine, New York, NY. She is currently a Senior Staff Scientist in the laboratory of Dr. Keith R. Jerome, in the Vaccine and Infectious Disease Division at the Fred Hutchinson Cancer Research Center, Seattle, WA. Her research efforts have focused on new therapeutic approaches to cure latent viral infection by targeted mutagenesis using designer rare cutting nucleases since the initiation of the project in 2008. These efforts have resulted in many new and exciting collaborative multidisciplinary projects spanning the fields of virology, immunology, structural biology, and cell biology. While the original work targeted the elimination of latent HIV infection, it is now expanded to other latent infectious agents, including herpes simplex virus, hepatitis B virus, and human papillomavirus.



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Nicholas D. Weber obtained his Ph.D. at the Universidad Autónoma de Madrid in Madrid, Spain, where he carried out research on nanoparticle delivery vectors for small interfering RNA (siRNA) to HIV-infected lymphocytes in the Laboratory of Molecular Immunology of Gregorio Marañón Hospital. He has been awarded internships at the National Institute of Allergy and Infectious Diseases of the NIH in Hamilton, Montana, where he worked with interactions between exogenous and endogenous retroviruses, and at the University of Montana Division of Biological Sciences to work with intracellular signal receptor transactivation and sorting in endosomes. He began his scientific career doing research in rechargeable satellite fuel cell degradation at the Aerospace Corporation in Los Angeles, CA, where he also obtained his bachelor's degree in Chemistry at Occidental College. He currently holds a postdoctoral fellowship at Fred Hutchinson Cancer Research Center, where he works with DNA-targeting enzymes as a way of potentially addressing hepatitis B virus infection.



Esther Mintzer received her B.S. from the University of Idaho, where she investigated nuclear pore complex degradation during rhinovirus infection in the laboratory of Dr. Kurt Gustin. She then worked at Pacific Northwest National Laboratory, conducting research in copper electrochemistry for the MAJORANA neutrinoless double-beta decay experiment. She is currently a Ph.D. student in microbiology at the University of Washington, working in the laboratories of Dr. Keith R. Jerome and Dr. David Baker. Her doctoral research is focused on computational design of homing endonucleases for use as HIV therapeutics.



Daniel Stone obtained a B.Sc in Biochemistry in 1997 and a Ph.D. in gene therapy in 2001 from the University of Manchester, United Kingdom. Subsequently, he moved to the United States and held positions as a Senior Fellow and then an Instructor at the University of Washington, Seattle. Dr. Stone has held positions as an Associate Specialist at the University of California, Berkeley, and an Associate Scientist at California Pacific Medical Center, San Francisco. He is currently a Staff Scientist at the Fred Hutchinson Cancer Research Center. Dr. Stone has been developing adenovirus and adeno-associated virus vectors for over 10 years, and he recently joined the group of Dr. Keith R. Jerome to help develop delivery systems for DNA-editing enzymes targeting latent viral infections.



Keith R. Jerome is Head of the Virology Division in the University of Washington Department of Laboratory Medicine and leads the combined program in Infectious Disease Sciences/Virology at the Fred Hutchinson Cancer Research Center. He received his M.D. and Ph.D. degrees from Duke University, and subsequently did a fellowship in virology at the University of Washington under the mentorship of Dr. Lawrence Corey. Dr. Jerome's clinical focus is on the diagnosis of viral infections and the role the laboratory can play in improved patient care. The aim of his research program is to use gene editing techniques to develop new therapeutic approaches to previously incurable viral infections, including HIV, hepatitis B virus, human herpesvirus, and human papillomavirus infections.

