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Review

HIV-1 evolution: frustrating therapies, but disclosing molecular mechanisms

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Replication of HIV-1 under selective pressure frequently results in the evolution of virus variants that replicate more efficiently under the applied conditions. For example, in patients on antiretroviral therapy, such evolution can result in variants that are resistant to the HIV-1 inhibitors, thus frustrating the therapy. On the other hand, virus evolution can help us to understand the molecular mechanisms that underlie HIV-1 replication. For example, evolution of a defective virus mutant can result in variants that overcome the introduced defect by restoration of the original sequence or by the introduction of additional mutations in the viral genome. Analysis of the evolution pathway can reveal the requirements of the element under study and help to understand its function. Analysis of the escape routes may generate new insight in the viral life cycle and result in the identification of unexpected biological mechanisms. We have developed *in vitro* HIV-1 evolution into a systematic research tool that allows the study of different aspects of the viral replication cycle. We will briefly review this method of forced virus evolution and provide several examples that illustrate the power of this approach.

Keywords: HIV-1 evolution; antiviral therapy; drug-resistance; molecular mechanism; virus replication; RNA structure

1. INTRODUCTION TO HIV-1 EXPERIMENTAL EVOLUTION

There is abundant evidence for HIV-1 evolution in diverse settings. In fact, HIV-1 evolution follows the principles of Darwinian evolution, which is a two-step process. The first step consists of the generation of genetic variants. In the second step, this material is exposed to phenotypic selection. The error-prone reverse-transcription process is the major cause of the rapid generation of HIV-1 variants, resulting in a mixed viral population of related genomes, also termed quasispecies (Munoz *et al.* 1993; Domingo & Wain-Hobson 2009). When allowed to replicate, the HIV-1 variant with the best fitness will outcompete the other variants. It is very important to note that the mutation step of evolution is completely independent of the selection step. Even though the virus population is eventually shaped by phenotypic selection criteria, the nucleotide substitution rates will largely determine the set of mutants available within the viral quasispecies for this selection process. As we will present different virus evolution strategies, the importance of both steps will become apparent.

We prefer to start evolution experiments with a plasmid carrying a complete and infectious DNA copy of the HIV-1 genome. The use of such an infectious molecular clone as input material allows one to unequivocally score newly acquired mutations, which provides an important benefit over the use of a virus stock, which is likely to harbor diverse viral variants. The molecular clone also allows mutation of sequence or structured elements in the viral genome to study their function in replication. We usually start an evolution experiment by transfection of the molecular clone into T cells. Whereas the original virus (wild-type; wt) will replicate rapidly, replication of the mutant virus may be reduced or undetectable, in which case we maintain the cells in culture by splitting when needed. A low level of ongoing virus replication in combination with the error-prone viral replication machinery will drive evolution, which may result in the emergence of variants with improved replication potential and a concomitant increase in the viral load. Once signs of virus replication are observed, the virus is passaged onto fresh cells. Initially, a large inoculum is used that in addition to the virus also contains virus-producing cells. In most experiments, the replication capacity of the virus gradually increases and the size of the inoculum can be decreased in subsequent passages, till fast-replicating variants have evolved that can be passaged cell-free. At each passage, infected cells are isolated and stored for sequence analysis of the integrated proviral genome. The mere selection of a specific mutation does not necessarily mean that it is responsible for the restored replication capacity. It is necessary to experimentally demonstrate

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that the acquired mutation caused the increased replication capacity. To do so, the evolved sequence is re-cloned into the viral genome and the replication potential of this variant is compared with that of the original mutant virus. A similar protocol can be used to select virus variants that are resistant to specific virus inhibitors (e.g. reverse transcriptase or protease inhibitors) or that have adapted to specific conditions (e.g. replication in a specific cell type).

2. THE HIV-1 MUTATION FREQUENCY AS DRIVER OF EVOLUTION

The mutation frequency of HIV-1 varies considerably for different kinds of mutations. For example, the G-to-A mutation is observed most frequently and is considered to be relatively easy to generate. In general, transversions (purine to pyrimidine or pyrimidine to purine mutations) are seen less frequently than transitions (purine to purine or pyrimidine to pyrimidine mutations; Keulen *et al.* 1996). Moreover, double mutations are more difficult than single mutations and therefore much less frequently observed (Berkhout *et al.* 2001; Berkhout & de Ronde 2004). Given the considerable HIV-1 population size, combined with the rapid replication kinetics and the high error rate during viral replication, it can be calculated that all variant genotypes with single nucleotide substitutions will be available in an infected individual (Coffin 1995), but it is less likely that complex substitution patterns are available in the initial virus pool.

The virus populations that are handled in *in vitro* experiments will usually be much smaller, which will limit the available genotypic variation. The use of a molecular HIV-1 clone as starting material will reduce the initial genetic variation to almost zero. Since mutation is a chance process, one should not put too much value on the mutational pattern in an individual culture. One should ideally analyse virus variants that emerge in multiple independent cultures, which may provide different answers to the same problem. In most cases, multiple evolution routes are observed that provide a glimpse of the sequence space available to support virus replication. However, the repeated selection of the same or similar escape viruses is also very informative, as it indicates that the problem can only be solved by that frequently observed mutation.

We elaborated on the concept of easy and difficult mutations in the field of antiviral drug resistance. If relatively difficult drug-resistance mutations are consistently observed at certain positions in the viral genome, we hypothesized that it is likely that easier nucleotide substitutions at that codon did not pass the fitness selection criteria (Keulen *et al.* 1996). A notable example is provided by changes observed at codon 215 in the reverse transcriptase (RT) gene in HIV-1-infected individuals treated with the nucleotide inhibitor AZT (zidovudine). Variants were selected with either a Thr215Phe or a Thr215Tyr substitution. Both mutations require two nucleotide changes (ACC → TTC and ACC → TAC, respectively). There are five alternative amino acid substitutions (Ile, Ala, Ser, Pro, Asn) that are in fact easier to generate than Thr215Phe, and 10 substitutions (Ile, Ala, Ser, Pro, Asn, Val, Met, Leu, Asp, Gly) that are easier to generate than Thr215Tyr. Yet, none of these alternative substitutions have been detected *in vivo* and *in vitro*, arguing that they do not provide AZT-resistance or that they are incompatible with RT enzyme function and virus replication. These combined results suggest that only aromatic residues at position 215 can provide the AZT-resistance phenotype, which was confirmed by a mutational analysis (Lacey & Larder 1994).

3. CLONAL EVOLUTION HIGHLIGHTS THE MUTATIONAL STEP

Virus replication will result in variants that form the quasispecies. Subsequent competition will result in the outgrowth of the fittest variant. Thus, a single evolved variant will be selected over time and other less optimal variants will be missed. We described a limiting dilution evolution approach that prevents competition between different emerging replication-competent virus variants, which allows the outgrowth of sub-optimal virus variants. We applied this protocol for the selection of previously unidentified HIV-1 variants that are resistant to the nucleoside RT inhibitor 3TC (lamivudine) (Keulen *et al.* 1997). In this approach, infected cells were cultured in the presence of 3TC. Instead of maintaining a large cell-virus culture, the cells were serially diluted shortly after infection. The large-culture studies and 3TC-therapy of HIV-1-infected persons had yielded only two types of 3TC-resistant variants, with either a Met184Ile (ATG to ATA) or Met184Val (ATG to GTG) mutation in the catalytic domain of RT. The limiting dilution protocol did not only yield these Met184Ile and Met184Val variants (19 and 4 out of 32 clones, respectively), but also the novel Met184Thr variant (ATG to ACG; 9 out of 32 clones). Indeed, this 184Thr variant does replicate poorly and will easily be lost owing to competition in a larger population setting.

When the forces of competition are nullified, the likelihood of generating a particular mutation determines how the virus evolves. The results of the 3TC-selection experiment are fully consistent with this idea, as simple transitions are observed more frequently than more difficult transversions, and no double mutations were scored. One will only see the less likely events when a large number of independent evolution cultures are analysed. Among the 19 Met184Ile variants, we observed 18 times the expected Ile variant owing to a transition (ATG to ATA), but the alternative Ile codon owing to a single transversion (ATG to ATT) was scored only once. In larger viral populations, many possible variants may be present or within mutational reach, but selection will shape the quasispecies by the outgrowth of a limited number of top-fit virus variants.

In real life, virus evolution can depend on a complex interplay of the mutation and selection steps. For instance, 3TC-treated patients may initially show the 184Ile variant, which is made by the most frequently occurring G-to-A transition (Berkhout *et al.* 2001).

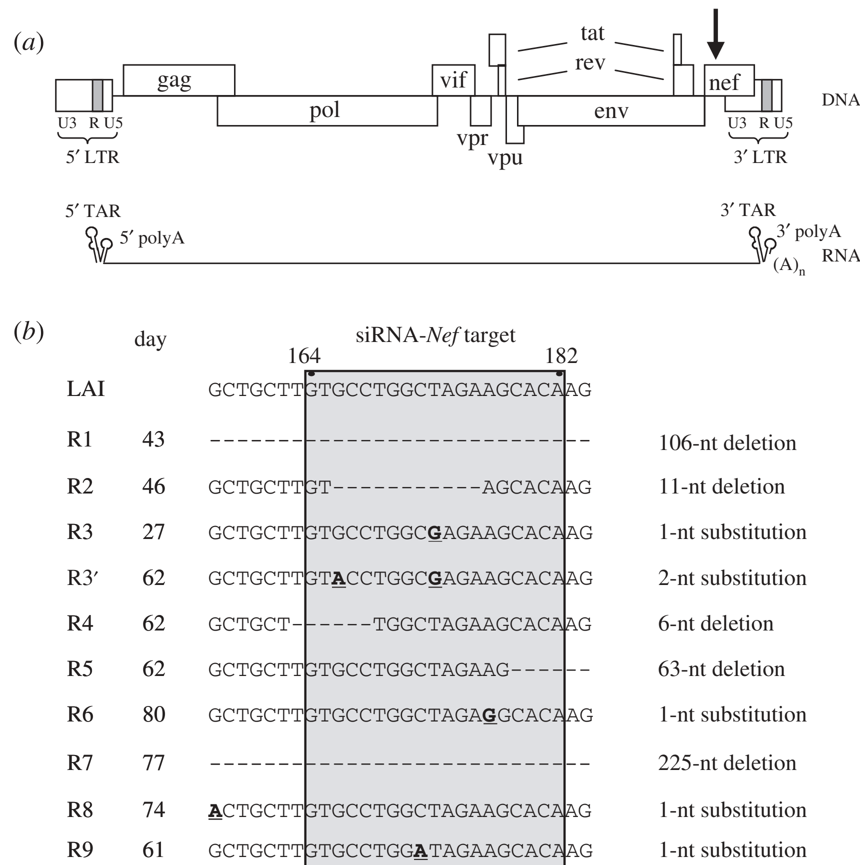

Figure 1. HIV-1 escape variants that resist siRNA-*Nef* inhibition. (*a*) Schematic of the HIV-1 proviral genome with the LTR region subdivided into the U3, R and U5 domains. Transcription starts at the first nucleotide of the 5′ R region and the RNA transcripts are polyadenylated at the last nucleotide of the 3′ R. Both the 5′ and the 3′ end of the RNA molecule can fold a TAR and polyA hairpin. The position of the siRNA-*Nef* target sequence is indicated with an arrow. (*b*) HIV-1 variants resistant to siRNA-*Nef* were selected in nine independent cultures. The *Nef* target sequence (nucleotides 164–182 of the *Nef* gene) and flanking sequences are shown for the wt (HIV-1 LAI molecular clone) and the evolved RNAi-resistant viruses (R1–R9). The day at which the escape variants were sequenced is indicated. Deletions are shown as dashes, substitutions are underlined and in bold. In the R1 virus, nucleotides 125–230 of the *Nef* gene are deleted. In the R5 virus, nucleotides 179–241 are deleted. In the R7 virus, we observed deletion of nucleotides 44–268 and a T269A substitution. Adapted from Westerhout *et al.* (2005). © copyright 2005 Westerhout *et al.*

This variant is subsequently outcompeted by the 184Val variant, which is more difficult to make via an A-to-G transition, but providing a subtly improved RT activity and viral fitness (Back *et al.* 1996). Thus, both mutation (Ile) and selection (Val) forces are visible in the evolution of 3TC-resistance.

4. FRUSTRATING ANTIVIRAL RNAi APPROACHES

RNA interference (RNAi) is an evolutionarily conserved process that provides the cell with an additional tool to regulate gene expression and to control infecting viruses (Berkhout & Haasnoot 2006; Obbard *et al.* 2009). RNAi may also be a powerful method for intracellular immunization against HIV-1 (Haasnoot *et al.* 2007). We and others demonstrated long-term inhibition of virus replication in human T cells that stably express small-interfering RNAs (siRNAs) directed against the HIV-1 genome (Banerjea *et al.* 2003; Boden *et al.* 2003; Das *et al.* 2004*a*; Lee *et al.* 2005; ter Brake *et al.* 2006, 2008*a*; von Eije *et al.* 2008, 2009; von Eije & Berkhout 2009). However, viral escape variants can emerge that are no longer inhibited (Boden *et al.* 2003; Das *et al.* 2004*a*). For example, in a study in which the HIV-1 *Nef* gene was targeted, the virus escaped from the imposed RNAi pressure in multiple independent cultures (Das *et al.* 2004*a*). The escape routes demonstrate the exquisite sequence-specificity of RNAi, as all escape variants—except for the R8 variant—carried a mutation within the 19-nucleotide target sequence (figure 1). We observed point mutations at different positions, but also deletions that partially or completely delete the target sequence, thus highlighting the chance process of the mutational event that triggers escape. Further analysis of the exceptional R8 mutation demonstrated that this escape route affects the local RNA structure, such that the target sequence is masked and not accessible for the RNAi machinery (Westerhout *et al.* 2005). This result underscores why we usually perform so many evolution experiments in parallel. As evolution is a chance process, one may need to probe multiple evolution tracks before the more exotic escape paths will be disclosed. Other large-scale escape studies indicated that the sequence variation induced by RNAi pressure mimics the natural variation in HIV-1 isolates, arguing that viral fitness

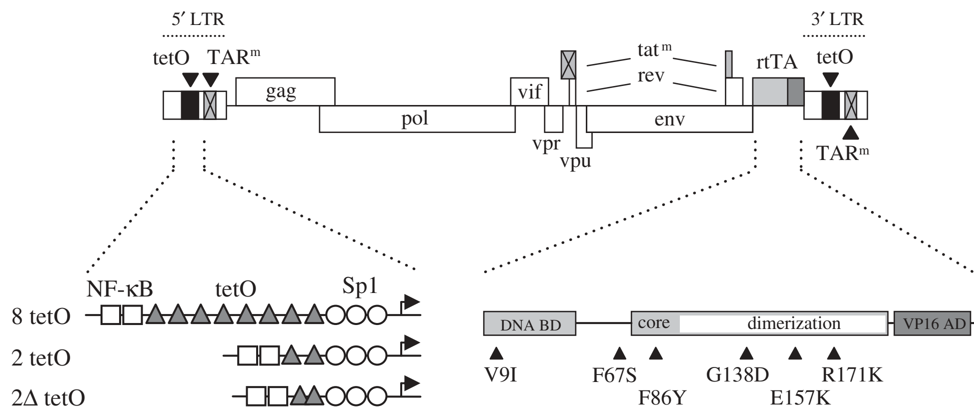

Figure 2. *In vitro* evolution of the doxycycline-dependent HIV-rtTA virus. In the HIV-rtTA virus, the Tat–TAR axis of transcription regulation has been inactivated by mutation of both Tat and TAR (tat^m and TAR^m, crossed boxes). Transcription and replication of the virus were made doxycycline-dependent by introduction of eight tetO elements in the LTR promoter region and replacing the *Nef* gene by the *rtTA* gene. This 248-amino acid protein is a fusion of the *E. coli* Tet repressor (TetR, which can be subdivided into a DNA-binding domain (BD) and a regulatory core domain with a dimerization surface) and the VP16 activation domain (AD) of herpes simplex virus. Administration of the doxycycline effector induces a conformational switch in the rtTA protein, which subsequently can bind to the tetO-LTR promoter region and activate transcription of the proviral genome. Thus, transcription and replication of HIV-rtTA are critically dependent on doxycycline. Evolution of HIV-rtTA resulted in modifications in the tetO promoter region (lower left panel; the original eight tetO and evolved two tetO and 2Δ tetO configurations are shown, see text for details) and in the *rtTA* gene (lower right panel). The black triangles indicate amino acid substitutions in rtTA that were observed upon evolution of HIV-rtTA in multiple, independent long-term cultures, and that were found to improve the transcriptional activity and doxycycline sensitivity of rtTA.

is a common and decisive determinant in both scenarios (ter Brake *et al.* 2008*b*; von Eije *et al.* 2008).

5. EVOLUTION OF HIV-1 VACCINES

Virus evolution experiments can play an important role in the safety testing of candidate vaccines. For instance, whereas AIDS vaccines based on a live-attenuated virus have shown promise in the SIV-macaque model, virus evolution studies revealed that such vaccines will likely be unsafe (Johnson & Desrosiers 1998; Mills *et al.* 2000; Whitney & Ruprecht 2004; Koff *et al.* 2006). The major safety concern is that a chronic infection is established and low-level replication of the attenuated virus may eventually lead to the selection of fitter and pathogenic virus variants (Baba *et al.* 1995, 1999; Whatmore *et al.* 1995; Chakrabarti *et al.* 2003; Hofmann-Lehmann *et al.* 2003). The Δ3 HIV-1 variant, which is attenuated by deletions in three non-essential genome segments, was considered a vaccine candidate because it seemed impossible that the virus could evolve to a pathogenic variant through repair of the deletions. However, we observed that this virus can regain replication fitness in prolonged cell culture infections (Berkhout *et al.* 1999). As expected, the deletions were not repaired. However, an intriguing sequence duplication was selected in the LTR promoter, which doubled the number of Sp1 binding sites from three to six and greatly improved virus replication. Multiplication or deletion of repeat sequence motifs is more frequently observed and seems to be a popular virus evolution strategy (Berkhout *et al.* 1999; Marzio *et al.* 2001; Leonard *et al.* 2008; Berkhout 2009).

Evolution experiments may also help in the development of more safe HIV-1 vaccine candidates. For example, we successfully used virus evolution to improve a doxycycline-dependent HIV-1 variant, which was developed as a novel approach to increase the safety of a live-attenuated vaccine strain (Verhoef *et al.* 2001). In this conditionally live virus, named HIV-rtTA, the Tat–TAR transcription activation mechanism was functionally replaced by the Tet-ON system for inducible gene expression (Baron & Bujard 2000; Gossen & Bujard 2001). Both Tat and TAR were inactivated through mutations at essential positions in the protein and RNA hairpin, respectively (figure 2). The Tet-ON system was integrated by insertion of the gene encoding the doxycycline-inducible rtTA *trans*-activator protein at the site of the *nef* gene and insertion of eight rtTA-binding tetO sites in the LTR promoter region. The original HIV-rtTA construct replicated poorly, but stably maintained the introduced components of the Tet-ON system and the mutations in Tat and TAR. Long-term culturing of HIV-rtTA resulted in the selection of greatly improved variants. Analysis of these evolved viruses revealed that the components of the Tet-ON system had been optimized (figure 2). The number of tetO elements was reduced to two and the spacing between the remaining motifs was subsequently reduced from 41 to 26 or 27 nucleotides (Marzio *et al.* 2001). This new spacing remarkably resembles the 29-nt spacing of the tetO elements in the *E. coli* Tn10 operon from which the Tet-ON components are derived. Further analysis showed that the new tetO configuration improved viral replication by fine-tuning of the LTR promoter activity (Marzio *et al.* 2002). Upon long-term culturing of HIV-rtTA, mutations were also observed in the rtTA protein that significantly improve its activity and doxycycline-sensitivity (Das *et al.* 2004*b*; Zhou *et al.* 2006*b*, 2007). We demonstrated that these evolved rtTA variants not only improve replication of

the conditionally live HIV-1 variant but also the performance of the Tet-ON system, which is widely used to regulate transgene expression. These new rtTA variants will be very useful in biological applications that require a more sensitive or active Tet-ON system. These studies demonstrate that the viral evolution strategy can be used to improve non-viral genes by making them an integral and essential part of the viral replication machinery.

In some extended cultures, we observed mutations at specific positions in rtTA that increased the background activity in the absence of doxycycline. These rtTA changes, which were always owing to one-nucleotide transitions, allowed virus replication in the absence of doxycycline. We therefore designed novel rtTA variants that require a more difficult nucleotide transversion or multiple simultaneous nucleotide substitutions to be converted to a doxycycline-independent variant (Zhou *et al.* 2006*a*,*c*). This higher genetic barrier did indeed block the undesired evolutionary routes of HIV-rtTA, which again illustrates the importance of the mutation step in viral evolution.

6. VIRUS EVOLUTION TO STUDY STRUCTURED RNA SIGNALS

We have successfully used evolutionary techniques to study important structured RNA motifs in the HIV-1 genome. Our initial studies addressed the TAR hairpin structure that is present at the 5′ and 3′ end of the viral RNA (figure 1*a*). The upper part of the 5′ TAR structure binds the viral transcriptional activator Tat protein and cellular co-factors, but no clear function for the lower stem region had been established. We reported that base substitutions in the lower stem region severely inhibited HIV-1 replication (Klaver & Berkhout 1994). Upon long-term culturing, better replicating viruses could be isolated with a great variety of genetic changes (point mutations, short deletions) that restored base-pairing of the lower stem region, which demonstrated that the stability of the TAR structure is essential for optimal HIV-1 replication.

Several TAR mutagenesis studies suggested that the TAR hairpin has other functions in HIV-1 replication in addition to its role in transcription, such as translation, dimerization, packaging and reverse-transcription of the viral RNA (Das *et al.* 2007 and references therein). Most of these studies were complicated by the fact that mutations in TAR have a dominant negative effect on viral transcription, which obscures other effects in the viral life cycle. We therefore recently reinvestigated this issue in mutation–evolution studies using the HIV-rtTA variant that does not need TAR for the activation of transcription (Das *et al.* 2007). Surprisingly, deletions in either the left or right side of the TAR stem dramatically impaired HIV-rtTA replication (A and B mutants in figure 3*a*), while a double mutant that combines these deletions replicated efficiently (AB mutant). Evolution of the single-side mutants resulted in nucleotide substitutions in TAR that restored the stability of the stem and thereby improved viral replication. The nearly complete deletion of the TAR sequence also reduced HIV-rtTA replication (mutant E in figure 3*a*,*b*). In long-term cultures of this mutant, we observed two different evolution routes in which the remaining TAR nucleotides were either removed (variant E I in figure 3*b*) or replaced by an unrelated stable hairpin (variant E II). Both evolved TAR-deleted variants replicate efficiently, which demonstrates that TAR has no essential second function in HIV-1 replication, at least *in vitro*.

Subsequent analyses revealed that the single-side deletions that opened TAR caused an unforeseen interaction between the remaining TAR nucleotides and nucleotides downstream of the polyA hairpin (Vrolijk *et al.* 2008). This interaction resulted in an extension of the polyA hairpin that—like TAR—is present at both the 5′ and 3′ end of the viral RNA (figure 1*a*). This interaction altered the conformation of the untranslated leader at the 5′ end of the viral RNA, which affected the processes of RNA dimerization and packaging (Vrolijk *et al.* 2008 and unpublished results). Moreover, stabilization of the polyA hairpin at the 3′ end of the viral RNA affected the process of polyadenylation, which further inhibited viral replication (Vrolijk *et al.* 2009). These studies demonstrate that mutational analysis of TAR is risky because it can induce unwanted side effects that affect non-related replication steps. These effects can easily be misinterpreted as providing evidence for secondary TAR functions.

We used similar virus evolution experiments to study other structured RNA signals in HIV-1 (e.g. polyA and SD hairpin; Das *et al.* 1997, 1999*a*; Abbink & Berkhout 2007), which eventually led to the description of an alternative HIV-1 RNA leader structure in which the polyA hairpin is opened to allow base-pairing with the DIS domain (Huthoff & Berkhout 2001; Abbink & Berkhout 2003; Ooms *et al.* 2004). The equilibrium between the two leader conformations may function as a riboswitch that is important in controlling RNA dimerization and packaging. Virus evolution approaches have also successfully been used by others to study RNA signals in the RNA bacteriophage MS2 (Olsthoorn & van Duin 1996; Klovins *et al.* 1997; Licis *et al.* 2000).

7. HELPING THE MUTATION STEP BY SEQUENCE RANDOMIZATION

The Systematic Evolution of Ligands by EXponential enrichment (SELEX) technique is a powerful method for the selection of molecules with unique properties (Breaker 2004). Whereas Darwinian evolution usually applies to living organisms over long periods of time, SELEX allows for the rapid *in vitro* evolution of functionally active nucleic acids from a pool of randomized sequences. We described an *in vivo* version of this nucleic acid evolution protocol in which selection and amplification take place inside living cells by means of HIV-1 replication (Berkhout & Klaver 1993). In brief, we generated a library of HIV-1 DNA genomes with random sequences in a particular genetic domain. This mixture of HIV-1 genomes was transfected in human T cells and the outgrowth of the fittest viruses was observed within two weeks of

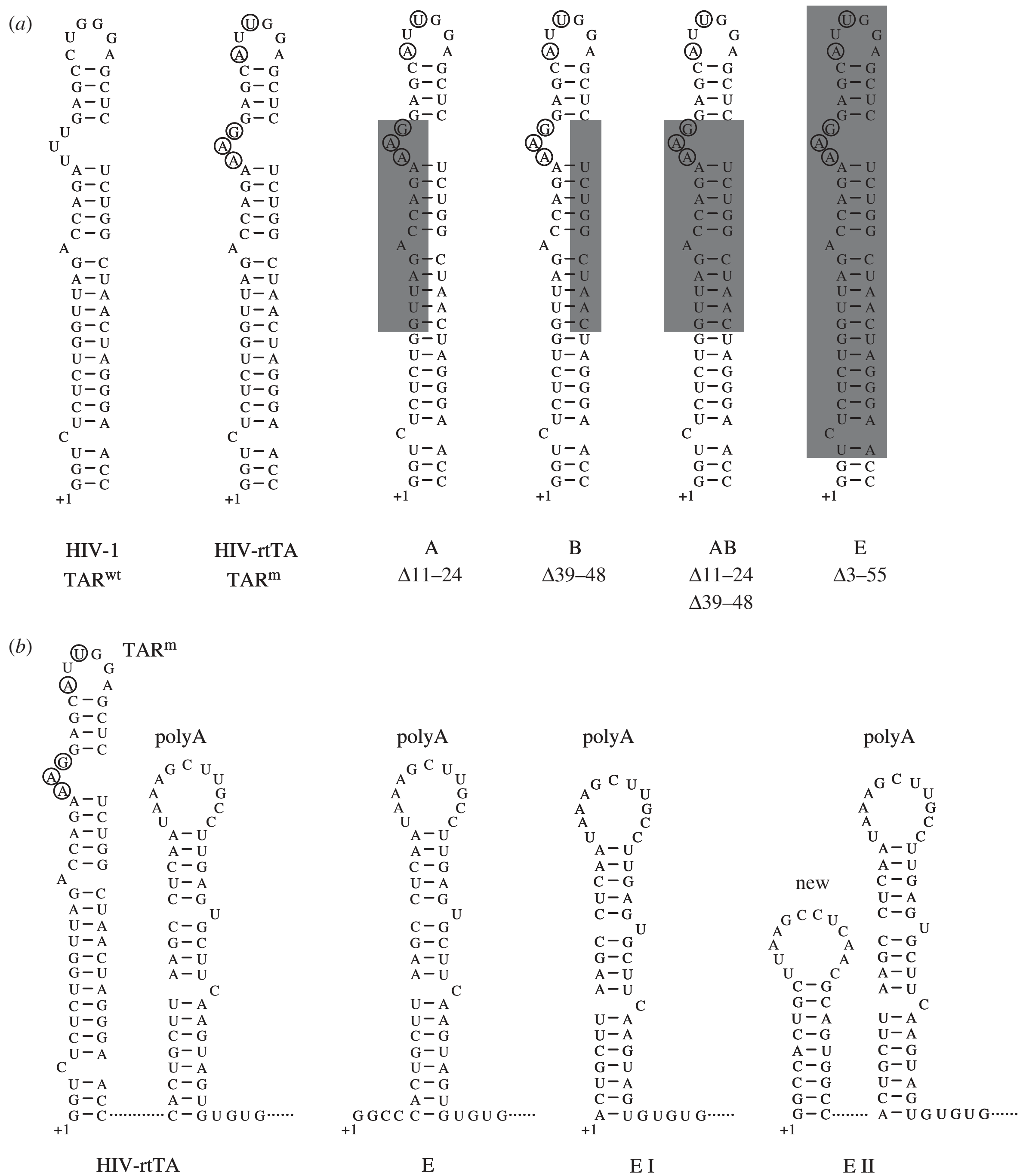

Figure 3. HIV-1 requires a stable hairpin structure at the 5′ end of its RNA genome. (*a*) The HIV-1 molecular clone LAI contains a 57-nt wild-type TAR hairpin (TAR^{wt}). In HIV-rtTA, which is based on LAI, TAR was inactivated by nucleotide substitutions in both the bulge and loop motifs (encircled in TAR^{m}). The TAR^{m} sequence was partially or nearly completely deleted (mutants A–E). The deleted nucleotides are boxed in grey. (*b*) Secondary structure of the 5′ end of the viral RNA of the HIV-rtTA variant, the E mutant and the evolved E I and E II variants.

viral replication. Compared with the laborious *in vitro* selection and amplification steps, much time and effort is saved in this *in vivo* approach. Moreover, adaptive changes may arise outside the randomized genome segment during virus replication due to the error-prone viral replication machinery. We used this approach to probe the sequence requirements for the HIV-1 TAR hairpin structure (Berkhout & Klaver 1993). We randomized the 3-nt bulge region to which the viral Tat transcriptional activator protein binds. Replication of this mixture of viruses resulted in the rapid selection of the wild-type bulge sequence UCU, which is also present in 80 per cent of the natural HIV-1 isolates. The method has also been successfully applied to study the replication of other retroviruses (Doria-Rose & Vogt 1998; Morris *et al.* 2002), hepatitis B virus (Rieger & Nassal 1995), plant viruses (Sun *et al.* 2005; Zhang & Simon 2005) and herpes simplex virus type 1 (Yoon & Spear 2004). Although many RNA viruses exist as a quasispecies of closely related but genetically distinct genotypes, their evolutionary potential is restricted because they probe only into a limited area of sequence space around the quasispecies. *In vivo*

SELEX provides the opportunity to sample a larger part of the sequence space by randomization of multiple nucleotides, yielding valuable information and new molecules with interesting properties.

8. SECOND-SITE MUTATIONS

In most mutation–evolution studies, HIV-1 will restore the function of the mutated element through sequence changes in this domain. The mutated virus can also acquire additional mutations outside the mutated region that improve viral replication. For example, in evolution studies with a Tat-inactivated HIV-1 variant, the virus acquired an additional mutation in another domain of the viral Tat protein. This second-site mutation partially restored Tat activity and viral replication, although a structural explanation is currently lacking (Verhoef & Berkhout 1999). Second-site mutations can also occur in other genome segments to impose a general replication improvement that is not related to the initial defect. For instance, we selected an up-mutation in the Envelope protein in studies with an HIV-1 variant that was translationally crippled through modification of the 5′ untranslated leader region (Das *et al.* 1998). Although it is attractive to think about a functional coupling of these two issues, further analysis revealed that the second-site mutation presented a general improvement of viral replication fitness that is not directly linked to the leader modifications (Das *et al.* 1999*b*). Indeed, this same Envelope mutation appeared later in a completely unrelated evolution study (Baldwin & Berkhout 2006). Some recent examples indicate that the misinterpretation of such a second-site mutation as a direct effect can become a major problem in the interpretation of HIV-1 evolution studies (Hache *et al.* 2008, 2009; Leonard *et al.* 2008; Berkhout 2009).

9. DISCUSSION

Several basic and applied research lines on HIV-1 were presented in which we used diverse virus evolution methods. These studies ranged from the selection of drug-resistant HIV-1 variants by virus evolution under selective pressure to the study of critical RNA hairpin motifs in the HIV-1 genome by means of forced evolution of virus mutants. These examples underscore the enormous potential of incorporating evolution methods in the design of virus experiments. It may therefore be surprising that only a few laboratories have used the virus evolution method in a systematic manner. One should obviously keep in mind the limitations of such *in vitro* HIV-1 studies, which will—among other things—miss the major selection pressure imposed by the adaptive immune system. A major advantage of virus evolution approaches over regular hypothesis-driven research methods is that one will frequently stumble on new phenomena that are disclosed in the physiologically relevant setting of replicating virus. For those researchers that are willing to listen to the virus, there will be many unforeseen discoveries that the virus is trying to teach us about. We have trained ourselves and our students to foster unexpected viral evolution routes because they can form the basis for truly new discoveries. This sometimes means that the scientist has to abandon the traditional way of doing science, in which a pre-existing model is tested by means of specific experiments. To be able to carefully listen to what the virus is telling us, it is preferable that the researcher starts with an unbiased view. This paradigm shift in how to perform scientific experiments may be the most difficult part of performing pioneering virus evolution studies.

REFERENCES

Abbink, T. E. M. & Berkhout, B. 2003 A novel long distance base-pairing interaction in human immunodeficiency virus type 1 RNA occludes the gag start codon. *J. Biol. Chem.* **278**, 11601–11611. (doi:10.1074/jbc.M210291200)

Abbink, T. E. & Berkhout, B. 2007 RNA structure modulates splicing efficiency at the HIV-1 major splice donor. *J. Virol.* **82**, 3090–3098. (doi:10.1128/JVI.01479-07)

Baba, T. W., Jeong, Y. S., Penninck, D., Bronson, R., Greene, M. F. & Ruprecht, R. M. 1995 Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* **267**, 1820–1825. (doi:10.1126/science.7892606)

Baba, T. W. *et al.* 1999 Live attenuated, multiply deleted simian immunodeficiency virus causes AIDS in infant and adult macaques. *Nat. Med.* **5**, 194–203. (doi:10.1038/8859)

Back, N. K. T., Nijhuis, M., Keulen, W., Boucher, C. A. B., Oude Essink, B. B., van Kuilenburg, A. B. P., Van Gennip, A. H. & Berkhout, B. 1996 Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J.* **15**, 4040–4049.

Baldwin, C. E. & Berkhout, B. 2006 Second site escape of a T20-dependent HIV-1 variant by a single amino acid change in the CD4 binding region of the envelope glycoprotein. *Retrovirology* **3**, 84. (doi:10.1186/1742-4690-3-84)

Banerjea, A., Li, M. J., Bauer, G., Remling, L., Lee, N. S., Rossi, J. & Akkina, R. 2003 Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. *Mol. Ther.* **8**, 62–71. (doi:10.1016/S1525-0016(03)00140-0)

Baron, U. & Bujard, H. 2000 Tet repressor-based system for regulated gene expression in eukaryotic cells: principles and advances. *Methods Enzymol.* **327**, 401–421. (doi:10.1016/S0076-6879(00)27292-3)

Berkhout, B. 2009 A new Houdini act: multiple routes for HIV-1 escape from RNAi-mediated inhibition. *Future Microbiol.* **4**, 151–154. (doi:10.2217/17460913.4.2.151)

Berkhout, B. & de Ronde, A. 2004 APOBEC3G versus reverse transcriptase in the generation of HIV-1 drug-resistance mutations. *AIDS* **18**, 1861–1863. (doi:10.1097/00002030-200409030-00022)

Berkhout, B. & Haasnoot, J. 2006 The interplay between virus infection and the cellular RNA interference machinery. *FEBS Lett.* **580**, 2896–2902. (doi:10.1016/j.febslet.2006.02.070)

Berkhout, B. & Klaver, B. 1993 *In vivo* selection of randomly mutated retroviral genomes. *Nucleic Acids Res.* **21**, 5020–5024. (doi:10.1093/nar/21.22.5020)

Berkhout, B., Verhoef, K., van Wamel, J. L. & Back, N. K. 1999 Genetic instability of live, attenuated human

immunodeficiency virus type 1 vaccine strains. *J. Virol.* **73**, 1138–1145.

Berkhout, B., Das, A. T. & Beerens, N. 2001 HIV-1 RNA editing, hypermutation and error-prone reverse transcription. *Science* **292**, 7. (doi:10.1126/science.292.5514.7a)

Boden, D., Pusch, O., Lee, F., Tucker, L. & Ramratnam, B. 2003 Human immunodeficiency virus type 1 escape from RNA interference. *J. Virol.* **77**, 11531–11535. (doi:10.1128/JVI.77.21.11531-11535.2003)

Breaker, R. R. 2004 Natural and engineered nucleic acids as tools to explore biology. *Nature* **432**, 838–845. (doi:10.1038/nature03195)

Chakrabarti, L. A., Metzner, K. J., Ivanovic, T., Cheng, H., Louis-Virelizier, J., Connor, R. I. & Cheng-Mayer, C. 2003 A truncated form of Nef selected during pathogenic reversion of simian immunodeficiency virus SIVmac239-Deltanef increases viral replication. *J. Virol.* **77**, 1245–1256. (doi:10.1128/JVI.77.2.1245-1256.2003)

Coffin, J. M. 1995 HIV population dynamics *in vivo*: implications for genetic variation, pathogenesis, and therapy. *Science* **267**, 483–489. (doi:10.1126/science.7824947)

Das, A. T., Klaver, B., Klasens, B. I. F., van Wamel, J. L. B. & Berkhout, B. 1997 A conserved hairpin motif in the R-U5 region of the human immunodeficiency virus type 1 RNA genome is essential for replication. *J. Virol.* **71**, 2346–2356.

Das, A. T., van Dam, A. P., Klaver, B. & Berkhout, B. 1998 Improved envelope function selected by long-term cultivation of a translation-impaired HIV-1 mutant. *Virology* **244**, 552–562.

Das, A. T., Klaver, B. & Berkhout, B. 1999*a* A hairpin structure in the R region of the human immunodeficiency virus type 1 RNA genome is instrumental in polyadenylation site selection. *J. Virol.* **73**, 81–91.

Das, A. T., Land, A., Braakman, I., Klaver, B. & Berkhout, B. 1999*b* HIV-1 evolves into a non-syncytium-inducing virus upon prolonged culture *in vitro*. *Virology* **263**, 55–69. (doi:10.1006/viro.1999.9898)

Das, A. T., Brummelkamp, T. R., Westerhout, E. M., Vink, M., Madiredjo, M., Bernards, R. & Berkhout, B. 2004*a* Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J. Virol.* **78**, 2601–2605. (doi:10.1128/JVI.78.5.2601-2605.2004)

Das, A. T., Zhou, X., Vink, M., Klaver, B., Verhoef, K., Marzio, G. & Berkhout, B. 2004*b* Viral evolution as a tool to improve the tetracycline-regulated gene expression system. *J. Biol. Chem.* **279**, 18776–18782. (doi:10.1074/jbc.M313895200)

Das, A. T., Harwig, A., Vrolijk, M. M. & Berkhout, B. 2007 The TAR hairpin of human immunodeficiency virus type-1 can be deleted when not required for Tat-mediated activation of transcription. *J. Virol.* **81**, 7742–7748. (doi:10.1128/JVI.00392-07)

Domingo, E. & Wain-Hobson, S. 2009 The 30th anniversary of quasispecies. Meeting on 'Quasispecies: past, present and future'. *EMBO Rep.* **10**, 444–448. (doi:10.1038/embor.2009.61)

Doria-Rose, N. A. & Vogt, V. M. 1998 *In vivo* selection of Rous sarcoma virus mutants with randomized sequences in the packaging signal. *J. Virol.* **72**, 8073–8082.

Gossen, M. & Bujard, H. 2001 Tetracyclines in the control of gene expression in eukaryotes. In *Tetracyclines in biology, chemistry and medicine* (eds M. Nelson, W. Hillen & R. A. Greenwald), pp. 139–157. Basel, Switzerland: Birkhäuser Verlag.

Haasnoot, J., Westerhout, E. M. & Berkhout, B. 2007 RNA interference against viruses: strike and counterstrike. *Nat. Biotechnol.* **25**, 1435–1443. (doi:10.1038/nbt1369)

Hache, G., Shindo, K., Albin, J. S. & Harris, R. S. 2008 Evolution of HIV-1 isolates that use a novel Vif-independent mechanism to resist restriction by human APOBEC3G. *Curr. Biol.* **18**, 819–824. (doi:10.1016/j.cub.2008.04.073)

Hache, G., Abbink, T. E., Berkhout, B. & Harris, R. S. 2009 Optimal translation initiation enables Vif-deficient human immunodeficiency virus type 1 to escape restriction by APOBEC3G. *J. Virol.* **83**, 5956–5960. (doi:10.1128/JVI.00045-09)

Hofmann-Lehmann, R., Vlasak, J., Williams, A. L., Chenine, A. L., McClure, H. M., Anderson, D. C., O'Neil, S. & Ruprecht, R. M. 2003 Live attenuated, nef-deleted SIV is pathogenic in most adult macaques after prolonged observation. *AIDS* **17**, 157–166. (doi:10.1097/00002030-200301240-00004)

Huthoff, H. & Berkhout, B. 2001 Two alternating structures for the HIV-1 leader RNA. *RNA* **7**, 143–157. (doi:10.1017/S1355838201001881)

Johnson, R. P. & Desrosiers, R. C. 1998 Protective immunity induced by live attenuated simian immunodeficiency virus. *Curr. Opin. Immunol.* **10**, 436–443. (doi:10.1016/S0952-7915(98)80118-0)

Keulen, W., Boucher, C. & Berkhout, B. 1996 Nucleotide substitution patterns can predict the requirements for drug-resistance of HIV-1 proteins. *Antiviral Res.* **31**, 45–57. (doi:10.1016/0166-3542(96)00944-8)

Keulen, W., Back, N. K. T., van Wijk, A., Boucher, C. A. B. & Berkhout, B. 1997 Initial appearance of the 184Ile variant in lamivudine-treated patients is caused by the mutational bias of the human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* **71**, 3346–3350.

Klaver, B. & Berkhout, B. 1994 Evolution of a disrupted TAR RNA hairpin structure in the HIV-1 virus. *EMBO J.* **13**, 2650–2659.

Klovins, J., Tsareva, N. A., de Smit, M. H., Berzins, V. & van Duin, J. 1997 Rapid evolution of translational control mechanisms in RNA genomes. *J. Mol. Biol.* **265**, 372–384. (doi:10.1006/jmbi.1996.0745)

Koff, W. C. *et al.* 2006 HIV vaccine design: insights from live attenuated SIV vaccines. *Nat. Immunol.* **7**, 19–23. (doi:10.1038/ni1296)

Lacey, S. F. & Larder, B. A. 1994 Mutagenic study of codons 74 and 215 of the human immunodeficiency virus type 1 reverse transcriptase, which are significant in nucleoside analog resistance. *J. Virol.* **5**, 3421–3424.

Lee, S. K. *et al.* 2005 Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. *Blood* **106**, 818–826. (doi:10.1182/blood-2004-10-3959)

Leonard, J. N., Shah, P. S., Burnett, J. C. & Schaffer, D. V. 2008 HIV evades RNA interference directed at TAR by an indirect compensatory mechanism. *Cell Host Microbe* **4**, 484–494. (doi:10.1016/j.chom.2008.09.008)

Licis, N., Balklava, Z. & van Duin, J. 2000 Forced retroevolution of an RNA bacteriophage. *Virology* **271**, 298–306. (doi:10.1006/viro.2000.0327)

Marzio, G., Verhoef, K., Vink, M. & Berkhout, B. 2001 *In vitro* evolution of a highly replicating, doxycycline-dependent HIV for applications in vaccine studies. *Proc. Natl Acad. Sci. USA* **98**, 6342–6347. (doi:10.1073/pnas.111031498)

Marzio, G., Vink, M., Verhoef, K., de Ronde, A. & Berkhout, B. 2002 Efficient human immunodeficiency virus replication requires a fine-tuned level of transcription. *J. Virol.* **76**, 3084–3088. (doi:10.1128/JVI.76.6.3084-3088.2002)

Mills, J., Desrosiers, R., Rud, E. & Almond, N. 2000 Live attenuated HIV vaccines: proposal for further research and development. *AIDS Res. Hum. Retroviruses* **16**, 1453–1461. (doi:10.1089/088922200750005976)

Morris, S., Johnson, M., Stavnezer, E. & Leis, J. 2002 Replication of avian sarcoma virus in vivo requires an interaction between the viral RNA and the TpsiC loop of the tRNA(Trp) primer. *J. Virol.* **76**, 7571–7577. (doi:10.1128/JVI.76.15.7571-7577.2002)

Munoz, J. L., Parks, W. P., Wolinsky, S. M., Korber, B. T. & Hutto, C. 1993 HIV-1 reverse transcriptase. A diversity generator and quasispecies regulator. *Ann. NY Acad. Sci.* **693**, 65–70. (doi:10.1111/j.1749-6632.1993.tb26257.x)

Obbard, D. J., Gordon, K. H., Buck, A. H. & Jiggins, F. M. 2009 The evolution of RNAi as a defence against viruses and transposable elements. *Phil. Trans. R. Soc. B* **364**, 99–115. (doi:10.1098/rstb.2008.0168)

Olsthoorn, R. C. L. & van Duin, J. 1996 Evolutionary reconstruction of a hairpin deleted from the genome of an RNA virus. *Proc. Natl Acad. Sci. USA* **93**, 12 256–12 261. (doi:10.1073/pnas.93.22.12256)

Ooms, M., Huthoff, H., Russell, R., Liang, C. & Berkhout, B. 2004 A riboswitch regulates RNA dimerization and packaging in human immunodeficiency virus type 1 virions. *J. Virol.* **78**, 10 814–10 819. (doi:10.1128/JVI.78.19.10814-10819.2004)

Rieger, A. & Nassal, M. 1995 Distinct requirements for primary sequence in the 5′- and 3′-part of a bulge in the hepatitis B virus RNA encapsidation signal revealed by a combination *in vivo* selection/*in vitro* amplification system. *Nucleic Acids Res.* **23**, 3909–3915. (doi:10.1093/nar/23.19.3909)

Sun, X., Zhang, G. & Simon, A. E. 2005 Short internal sequences involved in replication and virion accumulation in a subviral RNA of turnip crinkle virus. *J. Virol.* **79**, 512–524. (doi:10.1128/JVI.79.1.512-524.2005)

ter Brake, O., Konstantinova, P., Ceylan, M. & Berkhout, B. 2006 Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol. Ther.* **14**, 883–892. (doi:10.1016/j.ymthe.2006.07.007)

ter Brake, O., 't Hooft, K., Liu, Y. P., Centlivre, M., von Eije, K. J. & Berkhout, B. 2008*a* Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol. Ther.* **16**, 557–564. (doi:10.1038/sj.mt.6300382)

ter Brake, O., von Eije, K. J. & Berkhout, B. 2008*b* Probing the sequence space available for HIV-1 evolution. *AIDS* **22**, 1875–1877. (doi:10.1097/QAD.0b013e328309efe3)

Verhoef, K. & Berkhout, B. 1999 A second-site mutation that restores replication of a Tat-defective human immunodeficiency virus. *J. Virol.* **73**, 2781–2789.

Verhoef, K., Marzio, G., Hillen, W., Bujard, H. & Berkhout, B. 2001 Strict control of human immunodeficiency virus type 1 replication by a genetic switch: Tet for Tat. *J. Virol.* **75**, 979–987. (doi:10.1128/JVI.75.2.979-987.2001)

von Eije, K. J. & Berkhout, B. 2009 RNA-interference-based gene therapy approaches to HIV type-1 treatment: tackling the hurdles from bench to bedside. *Antivir. Chem. Chemother.* **19**, 221–233.

von Eije, K. J., ter Brake, O. & Berkhout, B. 2008 Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. *J. Virol.* **82**, 2895–2903. (doi:10.1128/JVI.02035-07)

von Eije, K. J., ter Brake, O. & Berkhout, B. 2009 Stringent testing identifies highly potent and escape-proof anti-HIV short hairpin RNAs. *J. Gene Med.* **11**, 459–467. (doi:10.1002/jgm.1329)

Vrolijk, M. M., Ooms, M., Harwig, A., Das, A. T. & Berkhout, B. 2008 Destabilization of the TAR hairpin affects the structure and function of the HIV-1 leader RNA. *Nucleic Acids Res.* **36**, 4352–4363. (doi:10.1093/nar/gkn364)

Vrolijk, M. M., Harwig, A., Berkhout, B. & Das, A. T. 2009 Destabilization of the TAR hairpin leads to extension of the polyA hairpin and inhibition of HIV-1 polyadenylation. *Retrovirology* **6**, 13. (doi:10.1186/1742-4690-6-13)

Westerhout, E. M., Ooms, M., Vink, M., Das, A. T. & Berkhout, B. 2005 HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res.* **33**, 796–804. (doi:10.1093/nar/gki220)

Whatmore, A. M., Cook, N., Hall, G. A., Sharpe, S., Rud, E. W. & Cranage, M. P. 1995 Repair and evolution of nef *in vivo* modulates simian immunodeficiency virus virulence. *J. Virol.* **69**, 5117–5123.

Whitney, J. B. & Ruprecht, R. M. 2004 Live attenuated HIV vaccines: pitfalls and prospects. *Curr. Opin. Infect. Dis.* **17**, 17–26. (doi:10.1097/00001432-200402000-00004)

Yoon, M. & Spear, P. G. 2004 Random mutagenesis of the gene encoding a viral ligand for multiple cell entry receptors to obtain viral mutants altered for receptor usage. *Proc. Natl Acad. Sci. USA* **101**, 17 252–17 257. (doi:10.1073/pnas.0407892101)

Zhang, J. & Simon, A. E. 2005 Importance of sequence and structural elements within a viral replication repressor. *Virology* **333**, 301–315.

Zhou, X., Vink, M., Berkhout, B. & Das, A. T. 2006*a* Modification of the Tet-On regulatory system prevents the conditional-live HIV-1 variant from losing doxycycline-control. *Retrovirology* **3**, 82. (doi:10.1186/1742-4690-3-82)

Zhou, X., Vink, M., Klaver, B., Berkhout, B. & Das, A. T. 2006*b* Optimization of the Tet-On system for regulated gene expression through viral evolution. *Gene Ther.* **13**, 1382–1390. (doi:10.1038/sj.gt.3302780)

Zhou, X., Vink, M., Klaver, B., Verhoef, K., Marzio, G., Das, A. T. & Berkhout, B. 2006*c* The genetic stability of a conditional-live HIV-1 variant can be improved by mutations in the Tet-On regulatory system that restrain evolution. *J. Biol. Chem.* **281**, 17084–17091. (doi:10.1074/jbc.M513400200)

Zhou, X., Symons, J., Hoppes, R., Krueger, C., Berens, C., Hillen, W., Berkhout, B. & Das, A. T. 2007 Improved single-chain transactivators of the Tet-On gene expression system. *BMC Biotechnol.* **7**, 6. (doi:10.1186/1472-6750-7-6)