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Review

The determination of HIV-1 RT mutation rate, its possible allosteric effects, and its implications on drug resistance

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Abstract: The high mutation rate of human immunodeficiency virus type 1 (HIV-1) plays a major role in treatment resistance from the development of vaccines to long-lasting drugs. In addressing the crux of the issue, various attempts to estimate the mutation rate of HIV-1 resulted in a large range of 10^{-5} - 10^{-3} errors/bp/cycle due to the use of different types of investigation methods. In this review, we discuss the different assay methods, their findings on the mutation rates of HIV-1 and how the location of these mutations can be further analyzed for their potential allosteric effects to reveal potentially new inhibitors with different pharmacodynamics that can be used to circumvent fast occurring HIV drug resistance. Given that HIV is one of the fastest mutating viruses, it is a good model for comprehensive study of its mutations that can give rise to much horizontal understanding towards overall viral drug resistance as well as emerging viral diseases.

Keywords: retroviruses; HIV-1; reverse transcriptase; mutation rate; drug resistance, allostery

1. Introduction

HIV-1 is a genetically diverse virus where multiple unique sequence isolates can originate within single patients [1–5]. This high genetic variability aids in the viral evasion from the host immune system, hindering therapy. Under selection pressure (such as antiretroviral drugs), these drug resistant mutations accumulate and emerge [6,7], rendering the treatments ineffective and also deterring the development of effective vaccines against HIV-1 [8].

Among the genetically retroviruses [9–11], HIV-1 is the most diverse [12–15], contributed by its high mutation rate, genetic recombination, and viral replication [6,16,17]. Genetic recombination, resulting from two single drug resistant strains, can give rise to HIV-1 virions with multiple drug resistance. [18–21]. The virus is estimated to produce 10^9 virions per day in an infected individual [22], and when coupled with high mutation rates, allow for numerous mutations to be generated in a few viral generations. Wei and colleagues showed that complete replacement of HIV-1 wild type strains with drug resistant strains occurred within 2 – 4 weeks after the antiretroviral treatment [6], demonstrating the impact of the viral mutational rate.

The error rates and mutant frequencies of HIV-1 reverse transcriptase (RT) are found to be in the range of 10^{-5} - 10^{-3} errors/bp/cycle and 10^{-4} – 10^{-2} mutants/clones respectively (Table 1). Compared to other retroviruses, HIV-1 possesses several folds higher mutation rates (reviewed by [23,24]), making it the ideal model for studying viral drug resistance and emerging infections that are result of cross-species from mutations. If HIV drug resistance emergence can be comprehensively understood, there is much horizontal understanding towards overall viral drug resistance and emerging viral infections (where mutations enable cross-species zoonotic infections). In this, the lack of consensus in mutation rates observed in HIV-1 hints of multiple factors and contributors involved (see Table 1 and 3).

In this review, we will discuss the experimental fidelity assays, their various mutation rates, and the potential of non-active site mutations working allosterically that may shed light on future interventions.

2. HIV-1 Reverse Transcriptase (RT)

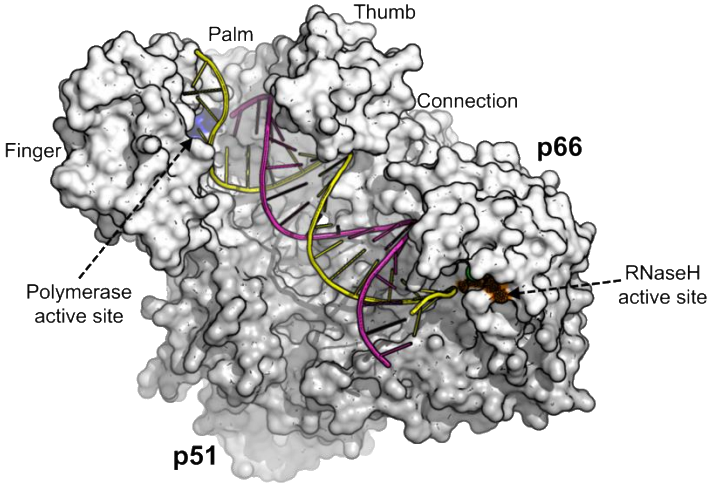

Figure 1. HIV-1 Reverse Transcriptase structure complexed with DNA (pdb 1T05)

HIV-1 RT lacks 3' exonuclease proofreading activity [25], contributing up to 68% mutations in cell-based assays during early stage replication (minus-strand synthesis and RNA transcription) and 32% during late stage replication (plus-strand synthesis and DNA repair) [26]. HIV-1 RT and host RNA polymerase II together have been found reported to account for around 52% mutation rate [27] while a study on the viral RNA from peripheral blood mononuclear cells (PBMCs) found the mutation rate to be 59.7%, and 2.0% in the DNA of the same PBMCs, possibly due to cytidine deaminases [28].

3. Comparison of Cell-free and Cell-based Fidelity Assays that affect the estimation of viral mutation rate

The fidelity of HIV RT would be best assessed directly on patient samples, but ethical and biosafety requirements are insurmountable obstacles for many small labs/institutes. Even when the obstacles are overcome, the virus would have undergone multiple generations of replication within the patient, making it nearly impossible to determine the fidelity of a single round of replication. Thus, various cell-free and cell-based approaches were developed, and we discuss their differences below.

3.1. Cell-free fidelity assays

In vitro or perhaps a better term "cell-free" assays to reflect the absence of HIV replication in cells in the experiments, allow for the reduction of confounding factors (e.g. a balanced dNTP pool), is an attempt to allow higher reproducibility. However, such reductionist methods also reduced several additional factors that may influence the fidelity of HIV RT, while subjecting the HIV target genes to high temperatures in PCR that could disrupt secondary structures of the nucleic acid.

One of the first cell-free fidelity assays used a synthetic polynucleotide template [29]. Typically, a homopolymer or an alternating copolymer, where errors can be determined using a mismatched radio-labelled nucleotide, is used. However, the homopolymer nucleotide templates do not accurately represent the events during synthesis of a natural heteropolymer template (such as a gene) [30], resulting in overestimation of mutation rates due to their repeating nature. Confounding factors such as the slippage of primers and stacking interactions between nucleotides are additional factors may also influence the fidelity of base substitution [29].

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Typically, reporter genes are used to provide visual differences between mutants and non-mutants. These reporter genes are commonly the α-complementing region of the lacZ gene (lacZα) and the DNA of bacteriophage ΦX174 (see Table 1 and 2). The earliest of such assays is the base reversion assay, measuring the error rate of RT on a single base [30] in the whole template and does not consider possible secondary structure influences by the RNA, overlooking the spectrum of mutations and any potential mutational hotspots caused by the HIV RT.

To address this limitation, a more common cell-free forward mutation assay is used with the lacZα reporter gene from the bacteriophage M13mp2 as a template [31]. HIV-1 RT would then be used to fill the gap, and the vector transformed into competent *E. coli* cells. Strands with gaps would lead to a partial or non-functional β-galactosidase protein, with clones appearing as white plaques on plates with X-gal and IPTG. In contrast, clones with functional β-galactosidase protein would appear as a dark blue plaque that can be sequenced. Using this method, mutation spectra and hotspots can potentially be determined.

Table 1. Error rates of HIV-1 RT measured in cell-free fidelity assays

Assay	RT Mutant	Vector	Reporter Gene / Template	Error Rate (x 10^{-4})	Reference
Base reversion	WT[1]	ΦX174 am3	Position 587 of ΦX174 am3, DNA	2.50	[32]
Base reversion	WT[1]	M13mp2	Position 89 of *lacZ*α coding sequence, DNA	0.55	[25]
Forward mutation			*lacZ*α, DNA	5.00 - 6.67	
Misincorporation	WT (HTLV-IIIB)	-	Polyadenylic acid	0.31 - 0.57	[33]
	WT (HIV[GUN-1])	-		0.26 – 0.38	
Base reversion	WT (HTLV-III)	ΦX174 am16	ΦX174 am16, DNA	1.43 - 2.00	[34]
Forward mutation	WT[1]	-	*lacZ*α, RNA	1.45	[35]
		-	M13mp2 (+), DNA	1.69	
Forward mutation	WT[1]	-	*env* V-1, DNA	1.90	[36]
		-	*env* V-1, RNA	2.00	
		-	*env* V-1, RNA/DNA	3.80	
Forward mutation	WT (BH10)	M13mp19 (CSIVM13B)	SIV_{agm}TYO-7 *env* (minus-strand), DNA	0.18	[37]
		M13mp19 (CSIVM13B)	*lacZ*α, DNA	0.53	
Forward mutation	WT (BH10)	M13mp2	*lacZ*α, DNA	0.45	[38]
	Q151M (BH10)			0.40	
	A62V/V75I/F77L/F116Y/Q151M (BH10)			0.23	
Forward mutation	WT (HXB2)	M13mp2	*lacZ*α, DNA	0.57	[39]
	E89G (HXB2)			0.41	
	M184V (HXB2)			0.36	
	E89G/M184V (HXB2)			0.81	
Forward mutation	WT (HXB2)	M13mp2	*lacZ*α, DNA	0.71*	[39,40]
	M184V (HXB2)			0.43*	

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Forward mutation	M184I (HXB2)	M13mp2	*lacZα*, DNA	0.17	[40]
Forward mutation	WT[1]	U-DNA Litmus 29 (Not)	*lacZα*, DNA	1.60	[41]
	Y115F[1]			1.00	
	Y115V[1]			4.70	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	0.57	[42]
	K65R (HXB2)			0.07	
	L74V (HXB2)			0.30	
Forward mutation	WT (NL4-3)	M13mp2	*lacZα*, DNA	0.63	[43]
	FE20 (NL4-3)			0.56	
	FE103 (NL4-3)			0.53	
Forward mutation	WT[1]	U-DNA Litmus 29 (Not)	*lacZα*, DNA	0.75	[44]
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	1.36	[45]
	WT (ESP49)			0.55	
	V75I (ESP49)			0.29	
Forward mutation	D433N (ESP49)	M13mp2	*lacZα*, DNA	0.14	[46]
	E478Q (ESP49)			0.1	
	V75I/D443N (ESP49)			0.14	
	V75I/E478Q (ESP49)			0.2	
	E478Q (BH10)			0.42	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	1.52	[47]
	WT (ROD)			1.00	
	K65R (ROD)			0.84	
	K65R/Q151M/M184V (ROD)			0.74	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, RNA	0.35	[48]
	WT (ESP49)			0.27	
	K65R (ESP49)			0.26	
	K65R/V75I (ESP49)			0.25	

[1]Lab strain of HIV-1 RT used was not mentioned

Table 2. Mutant frequencies of HIV-1 RT measured in cell-free fidelity assays

Assay	RT Mutant	Vector	Reporter Gene / Template	Mutant Frequency (x 10^{-4})	Reference
Base reversion	WT[1]	M13mp2	Position 89 of *lacZα* coding sequence, DNA	1.00	[25]
Forward mutation	WT[1]	M13mp2	*lacZα*, DNA	340 - 460	
Forward mutation	WT[1]	M13mp2	*lacZα*, DNA	390.00	[49]
Base reversion	WT[1]	pTZ18R	ΦX174 am16, RNA	26.00	[50]

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			ΦX174 am16, DNA	26.00	
Forward mutation	WT[1]	M13mp2	*lacZα*, DNA	340 - 540	[51]
			lacZα, RNA	91 - 210	
Forward mutation	WT[1]	M13mp2	*lacZα*, RNA	40.70	[35]
		pBluescript SK+	M13mp2, (+) DNA	47.30	
Forward mutation	WT[1]	M13mp2	pseudowild type 1 (pwt1) *lacZα*, DNA	490	[52]
			pseudowild type 2 (pwt2) *lacZα*, DNA	450	
			lacZα, DNA	500	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	200	[53]
	D256A (HXB2)			240	
	Q258A (HXB2)			390	
	K259A (HXB2)			300	
	L260A (HXB2)			230	
	G262A (HXB2)			880	
	K263A (HXB2)			290	
	W266A (HXB2)			640	
	Q269A (HXB2)			510	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	210	[54]
	G262A (HXB2)			860	
	W266A (HXB2)			630	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	160	[55]
	R277A (HXB2)			140	
	Q278A (HXB2)			190	
	L279A (HXB2)			150	
	C280A (HXB2)			300	
	K281A (HXB2)			140	
	L282A (HXB2)			120	
	R284A (HXB2)			170	
	G285A (HXB2)			160	
	K287A (HXB2)			120	
Forward mutation	WT (BH10)	M13mp19 (CSIVM13B)	SIV_{agm}TYO-7 *env* (minus-strand), DNA	31.40	[37]
			lacZα, DNA	60.90	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	232	[56]
	D76V (BH10)			26	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	64.00	[38]
	Q151M (BH10)			55.00	

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	A62V/V75I/F77L/F116Y/Q151M (BH10)			31.00	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	86.00	[39]
	E89G (HXB2)			62.60	
	M184V (HXB2)			55.30	
	E89G/M184V (HXB2)			123.00	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	97*	[39,40]
	M184V (HXB2)			59*	
Forward mutation	M184I (HXB2)	M13mp2	*lacZα*, DNA	24	[40]
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	250	[57]
	R78A (BH10)			28	
Base reversion	WT (HXB2)		TGA codon (position 87-89) in lacZα, DNA	2.2	[58]
	R72A (HXB2)			82	
One-nucleotide deletion reversion	WT (HXB2)		TTTT run in M13mp2 DNA	32	
	R72A (HXB2)			1.6	
Forward mutation	WT (HXB2)		*lacZα*, DNA	210	
	R72A (HXB2)			340	
Forward mutation	WT[1]	U-DNA Litmus 29 (Not)	*lacZα*, DNA	278.00	[41]
	Y115F[1]			175.00	
	Y115V[1]			82.00	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	192	[59]
	L74V (BH10)			55	
	E89G (BH10)			96	
	M184V (BH10)			228	
	Y183F (BH10)			303	
	Y115A (BH10)			763	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	86	[42]
	K65R (HXB2)			10.6	
	L74V (HXB2)			50.5	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	261	[60]
	Q151N (BH10)			20	
	K154A (BH10)			125	
Forward mutation	WT (NL4-3)	M13mp2	*lacZα*, DNA	86.00	[43]
	FE20 (NL4-3)			77.00	
	FE103 (NL4-3)			74.00	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	97	[61]
	F61A (HXB2)			8.3	

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Forward mutation	V184I (HXB2)	M13mp2	*lacZα*, DNA	30	[62]
Forward mutation	E89K (HXB2)	M13mp2	*lacZα*, DNA	77	[63]
	E89V (HXB2)			64	
	E89S (HXB2)			53	
Forward mutation	WT (HXB2)	M13mp2	*lacZα*, DNA	97	[64]
	T69S-AG (HXB2)			20	
	T69S-SG (HXB2)			12	
	T69S-SS (HXB2)			24	
	A62V/T69S-AG/L210W/R211K/L214F/T215Y			8.5	
	A62V/T69S-SG/L210W/R211K/L214F/T215Y			19	
	A62V/T69S-SS/L210W/R211K/L214F/T215Y			11	
	M41L/T69S-AG/L210W/R211K/L214F/T215Y			6.3	
	M41L/T69S-SG/L210W/R211K/L214F/T215Y			5.9	
Forward mutation	WT[1]	U-DNA Litmus 29 (Not)	*lacZα*, DNA	130.00	[44]
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	206	[65]
	V75A (BH10)			281	
	V75F (BH10)			112	
	V75I (BH10)			69.6	
Base reversion	WT (BH10)			27	
	V75I (BH10)			7.8	
Forward mutation	WT (ESP49)	M13mp2	*lacZα*, DNA	83.1	[45]
	V75I (ESP49)			43.4	
Forward mutation	K65R (ESP49)	M13mp2	*lacZα*, DNA	7.7	[66]
	K65R/V75I (ESP49)			8.9	
	R78A (ESP49)			5.9	
Forward mutation	WT (ESP49)	M13mp2	*lacZα*, DNA	96	[46]
	D433N (ESP49)			19.8	
	E478Q (ESP49)			13.5	
	V75I/D443N (ESP49)			18.2	
	V75I/E478Q (ESP49)			29.1	
	WT (BH10)			113.4 - 132.3	

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	E478Q (BH10)			57.6	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	199.00	[47]
	WT (ROD)			124.20	
	K65R (ROD)			117.90	
	K65R/Q151M/M184V (ROD)			103.10	
Forward mutation	WT (BH10)	M13mp2	*lacZα*, DNA	40.50	[48]
	WT (ESP49)			34.5	
	K65R (ESP49)			29.5	
	K65R/V75I (ESP49)			29.3	

[1]The lab strain of HIV-1 RT used was not mentioned

3.2. Cell-based fidelity assays

In vivo or "cell-based" assays partially re-create the natural environment that include factors such as host and viral proteins lacking in cell-free assays. These assays are less reproducible due to varied confounding factors when using different cells, and the assays typically utilize HIV shuttle vectors containing the HIV genes and reporter genes that are transfected into mammalian cells for a single round of replication, followed by transfer into suitable hosts (e.g. *E. coli*) for selection of mutants [67].

However, in both cell-based and cell-free assays, silent mutations are not detected, possibly leading to an underestimated fidelity of HIV RT.

Table 3. Error rates of HIV-1 RT measured in cell-based fidelity assays

Assay	RT Mutant	Vector	Reporter Gene / Template	Error Rate (x 10^{-4})	Reference
Forward mutation	WT (NL4-3)	HIV-1 vector (HIV shuttle 3.12 & 5.1)	*lacZα*, DNA	0.34	[67]
Forward mutation	WT (NL4-3)	HIV-1 vector (HIV shuttle 3.12)	*lacZα*, DNA	0.40	[68]
Forward mutation	WT (NL4-3)	HIV-1 vector (HIV shuttle 3.12 *vpr*$^+$)	*lacZα*, DNA	0.30	[69]
		HIV-1 vector (HIV shuttle 3.12 *vpr* ATG$^-$)		1.20	
Forward mutation (SSCP)	WT (HXB2)	HIV-1 vector (pHIV-gpt)	HIV-1 LTR, DNA	0.92	[27]
	WT (NL4-3)	HIV-1 vector (NL4-3gpt)		0.79	
Forward mutation	WT (NL4-3)	HIV-1 vector (pNL4-3deltaΔ +cass)	*tk*, DNA	0.22	[70]
Forward mutation	WT (NL4-3)	HIV-1 vector (pNL4-3 HIG)	U373-MAGI-X4 cells, DNA	6.90	[71]
Forward mutation	WT (NL4-3)	HIV-1 vector (pSICO-LZF)	*lacZα*, DNA	0.22	[72]
		HIV-1 vector (pSICO-LZR)		0.17	
Forward mutation	WT (NL4-3)	HIV-1 vector (pSICO-LZF/R)	*lacZα*, DNA	0.14	[73]
	Y115F (NL4-3)			0.37	
	Q151M (NL4-3)			0.17	
	M184I (NL4-3)			0.21	
	M184V (NL4-3)			0.18	

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Forward mutation	WT (HX2B2)	HIV-1 vector (pSDY-dCK)	HIV *env*, RNA	0.36	[74]
			HIV *Int-vif-vpr*, RNA	0.75	

Table 4. Mutant frequencies of HIV-1 RT measured in cell-based fidelity assays

Assay	RT Mutant	Vector	Reporter Gene / Template	Mutant Frequency (x 10^{-4})	Reference
Forward mutation	WT (NL4-3)	HIV-1 vector (HIV shuttle 3.12)	*lacZα*, DNA	44	[67]
		HIV-1 vector (HIV shuttle 5.2)		42	
Forward mutation	WT (NL4-3)	HIV-1 vector (HIV shuttle 3.12)	*lacZα*, DNA	50	[68]
Forward mutation	WT (NL4-3)	HIV-1 vector (HIV shuttle 3.12 *vpr* ATG-)	*lacZα*, DNA	150	[69]
		HIV-1 vector (HIV shuttle 3.12 *vpr* A30F)		140	
		HIV-1 vector (HIV shuttle 3.12 *vpr*+)		40	
Forward mutation	WT (NL4-3)	HIV-1 vector	*lacZα*, DNA	1500 – 1510[2]	[75]
	K65R (NL4-3)			450[2]	
	D67N (NL4-3)			1490[2]	
	K70R (NL4-3)			1470[2]	
	L74V (NL4-3)			1120[2]	
	D76V (NL4-3)			590 – 600[2]	
	R78A (NL4-3)			420 – 430[2]	
	E89G (NL4-3)			120[2]	
	Y115A (NL4-3)			3400 – 3480[2]	
	Q151N (NL4-3)			250 – 280[2]	
	K154A (NL4-3)			1520[2]	
	F227A (NL4-3)			930[2]	
	W229A (NL4-3)			720[2]	
	Y501W (NL4-3)			4300[2]	
	I505A (NL4-3)			1410[2]	
	D76V/R78A (NL4-3)			150[2]	
	R78A/Q151N (NL4-3)			110[2]	
	Y115A/Q151N (NL4-3)			1050[2]	
Forward mutation	WT (NL4-3)	HIV-1 vector (HIV shuttle 3.12)	*lacZα*, DNA	1490	[62]
	V148I (NL4-3)			390	
	Q151N (NL4-3)			260	
	WT (NL4-3)	HIV-1 vector (pSICO-LZF/R)	*lacZα* (F), DNA	38	[72]

Forward mutation		HIV-1 vector (pSICO-LZF/R)	*lacZα* (R), DNA	21.8	
		HIV-1 vector (pNLZeoIN-R-E-.LZF/R)	*lacZα* (F), DNA	21.7	
		HIV-1 vector (pNLZeoIN-R-E-.LZF/R)	*lacZα* (R), DNA	18.2	
Forward mutation	WT (NL4-3)	HIV-1 vector (pSICO-LZF/R)	*lacZα*, DNA	21.98	[73]
	Y115F (NL4-3)			55.91	
	Q151M (NL4-3)			25.69	
	M184I (NL4-3)			31.9	
	M184V (NL4-3)			27	

[2]Mutant frequency was calculated as mutants/cycle

5. Further studies of HIV-1 RT on HIV-1 Genes

When considering the mutation rates of HIV-1 RT and the emergence on HIV, the locations where these mutations occur are important to understand emerging drug resistance [76] as well as potential understanding to how viruses can jump species [77,78]**.** Although the mutation rate of HIV-1 RT has been widely studied, majority of the studies are performed using the reporter gene lacZα as its template [39–45,48,58,79], resulting in a major gap of understanding on how HIV-1 RT mutates HIV-1 genes directly.

We found only two studies that worked on HIV templates. One was on the HIV-1 *env* gene [36,74] (see Table 5) by Ji & Loeb in cell-free assays to show the error rate in DNA (1.90×10^{-4}), RNA (2.00×10^{-4}) and RNA/DNA (3.80×10^{-4}), that is comparable to previous M13mp2 forward assay using the lacZα gene as template in DNA (1.69×10^{-4}) and RNA (1.45×10^{-4}) [36]. The other study, by Geller and colleagues, investigated the HIV *env* and *int-vir-vpr* RNA, with rates at 0.36×10^{-4} and 0.75×10^{-4} respectively [74]. Ji & Loeb found that mutations produced by HIV RT *in vitro* partially correlated with mutations observed in AIDS patients, demonstrating the contribution of phenotypic selection during HIV-1 infection. On the other hand, Geller and colleagues reported that changes in sequence context and secondary structure controlled the activity of cytidine deamination and fidelity of HIV RT.

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Table 5. Percentages of nucleotide mutations of HIV-1 RT on HIV-1 gene and LacZα template

Reported percentages were calculated using previous research, with exact reported numbers indicated in parentheses.

Template	Base substitutions												Frameshifts		Others	Reference
	Transversions								Transitions				Insertions	Deletions		
	A -> C	C -> A	A -> T	T -> A	C -> G	G -> C	G -> T	T -> G	G -> A	A -> G	C -> T	T -> C				
HIV-1 *env* V-1, DNA	0	0	1.59 (1)	1.59 (1)	1.59 (1)	0	4.76 (3)	6.35 (4)	9.52 (6)	26.98 (17)	15.87 (10)	4.76 (3)	23.81 (15)	3.17 (2)	0	
HIV-1 *env* V-1, DNA/RNA	7.55 (4)	0	3.77 (2)	3.77 (2)	0	0	16.98 (9)	0	3.77 (2)	20.75 (11)	16.98 (9)	13.21 (7)	7.55 (4)	5.66 (3)	0	[36]
HIV-1 *env*	27.88 (29)								46.15 (48)	15.38 (16)			8.65 (9)	1.92 (2)	0	
HIV-1 *int-vif-vpr*	19.61 (20)								50.98 (52)	22.55 (23)			4.90 (2)	1.96 (2)	0	[74]
LacZα, RNA	-	-	-	-	-	10.64 (5)	23.40 (11)	-	0	-	31.91 (15)	2.13 (1)	14.89 (7)		17.02 (8)	[35]
LacZα, DNA	0	31.53 (70)	0	30.63 (68)	0.45 (1)	0.45 (1)	0	0.90 (2)	12.61 (28)	3.60 (8)	4.95 (11)	0	14.86 (33)		0	[41]
LacZα, DNA	0.57 (1)	11.93 (21)	0	9.66 (17)	0	2.27 (4)	0	0.57 (1)	45.45 (80)	6.25 (11)	7.39 (13)	1.14 (2)	10.23 (18)		4.55 (8)	[44]

6. Emerging drug resistance mutations considered as allosteric mutation hotspots

Many anti-HIV drugs target key viral enzymes [80], e.g. reverse transcriptase, integrase, and protease. Therefore, occurrences of mutations hotspots within HIV-1 genes are essential in understanding and the designing of new drugs to overcome viral resistance.

Allosteric effects can underlie both drug resistance and inhibition mechanisms that can be explored for novel classes of drugs to develop novel classes of reverse transcriptase inhibitors (RTIs) [81], integrase inhibitors [82], and protease inhibitors (PIs) [83]. For example, non-nucleoside RTIs are known to bind non-competitively to an allosteric site on p66 subunit to cause structural changes to the RT polymerase active site, hindering DNA polymerization. Studies suggested the RT p51 subunit, in addition to p66, to be also involved in allosteric couplings upon inhibitor binding [81,84]. Allosteric integrase inhibitors can impair the binding of integrase and the cellular cofactor LEDGF/p75 during HIV-1 replication and induce aberrant integrase multimerization [82]. Similarly, a potential allosteric modulator of HIV-1 protease was found to bind to an allosteric site at the protease flaps and equipotently inhibit both wild-type and certain drug resistant variants [83].

Since viral mutations were found clinically to emerge not only at the direct drug binding sites but also at distant regions of these enzymes [85], these mutations may exhibit allosteric effect to active sites. For example, HIV Gag [86], can have non-cleavage site mutations that exhibited allosteric communications to the cleavage sites [87] associated with protease inhibitor resistance [76] even in the absence of mutations in the cleavage sites directly.

Given limited and cost-inefficient experiments to explore allosteric mutations, allosteric signaling map [88] to computationally screen single point mutations and quantify possible allosteric communications between protein regions, e.g. for several HIV-1 proteins (Figure 2), facilitates quick mutation hotspot identifications. Asymmetrical effects were observed in HIV-1 RT structure, where mutated p51 could potentially cause varying (de)stabilization effects on p66, but not in reverse (Figure 2A). In addition, allosteric free energies ($\Delta\Delta g$) were estimated to quantify the residual allosteric signaling on the DNA polymerase active site (Figure 2B), where single mutations stabilized ($\Delta\Delta g < 0$) or destabilized($\Delta\Delta g > 0$) the active site. As a result, several possible RT mutation hotspots, which may potentially destabilize the DNA polymerase active site, were found on the thumb domain of p66 and on p51 (highlighted in red spheres and red dash ovals in Figure 2B). Since there has been no known inhibitor targeting p51, this suggests that new allosteric drugs can be designed to target the mutation hotspots on p51.

On the other hand, symmetrical allosteric communications between domains were observed for HIV-1 integrase and protease [89,90] likely due to the homo-multimerization (Figure 2A). The detected mutations hotspots on these two targets are on the C-terminal domain (CTD) of integrase and at the "ears" regions of protease (Figure 2B). For the Gag protein, allosteric communications were found to occur between several emerging non-cleavage site and cleavage site mutations to influence proteolysis and hence compensate for decreased viral fitness [87]. Several such non-cleavage hotspot mutations (e.g. on MA, CA, and p6) were also previously found *in vitro* in HIV-1 Gag to support these observations [91].

It is likely that screening of allosteric effects of mutation sites and hotspots may reveal additional targets within the targets that may allow pre-emptive drug design against HIV [76].

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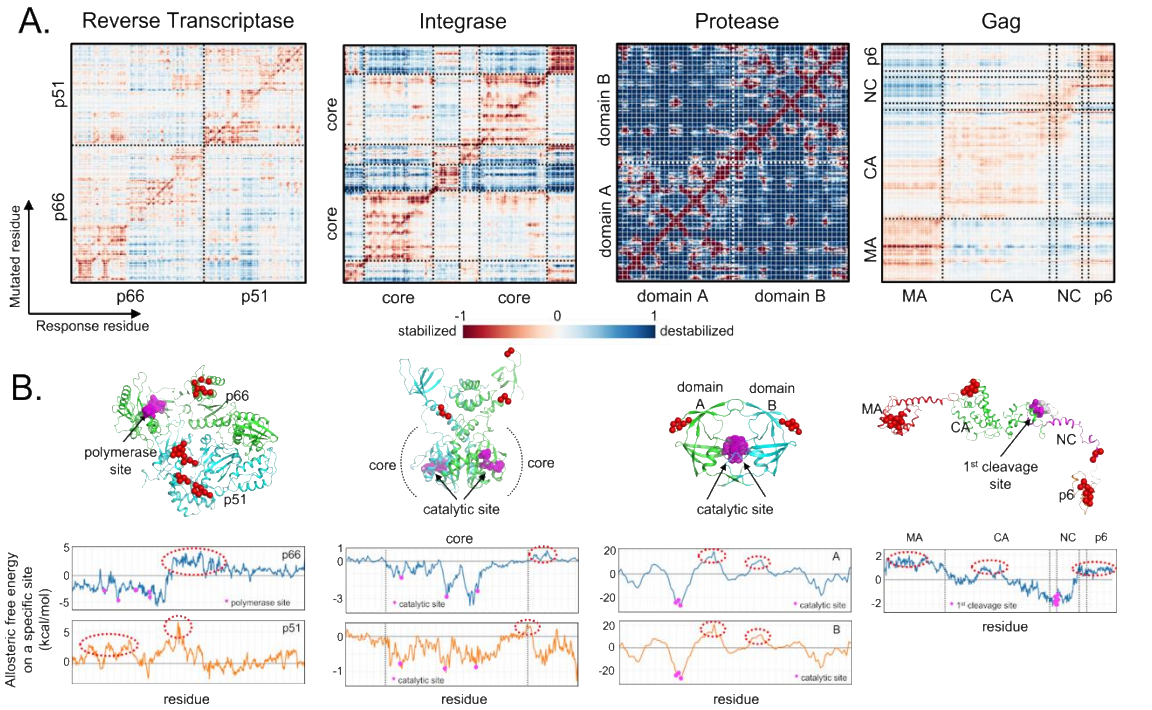

Figure 2. Underlying allosteric mechanisms were found in several HIV-1 proteins, from which it facilitates mutation hotspot detections. (**A**) Allosteric signaling maps (single point mutation screening) of HIV-1 Reverse Transcriptase, Integrase, Protease, and Gag. Structural presentation using RT (pdb 3T19), IN (reconstructed from pdb 1K6Y and 1EX4), PR (pdb 2PC0), and Gag model from Su et al. [87]; (**B**) Allosteric free energies (ΔΔg) on specific catalytic or cleavage sites were estimated based on individual perturbation at single residues (x-axis) to demonstrate the resulting stabilizing (ΔΔg < 0) or destabilizing (ΔΔg > 0) effect onto according sites of these proteins. The possible mutation hotspots, which may potentially destabilize the sites of interest, are highlighted in red spheres and red dash ovals.

7. Need for Biologically Similar Assays and its Implications on Drug Resistance

Current experimental work propose controlling viral mutation rates as a form of antiretroviral strategy [24]. In addition to controlling viral mutation rate of HIV-1 RT, determining mutational hotspots and rates of drug targets allow for the development and pre-emptive design of novel drugs. Although HIV-1 RT mutation rates have already been intensively studied, there are still gaps in studying the mutations on HIV genes, especially those that focus on the rate and types of mutation (e.g. substitution, deletion and insertion) and specific nucleotide changes (e.g. A to G) on other specific HIV-1 proteins (such as Gag, Protease, etc.). A target or region with lower mutation rates could be a better druggable site, especially when considering the trade-off with viral fitness. In the case of M184V resistance mutation in HIV-1 RT, it is known to increase fidelity, impair viral fitness, and increase hyper-sensitization to NRTIs (such as amprenavir and efavirenz) [92]. Thus, such examples demonstrate that it is possible to leverage on such features alongside structural understanding (e.g. in [76]) towards combinatorial therapies that target the active site using existing inhibitors, and the potential emerging mutational sites to tighten the deadlock.

While the fidelity of HIV RT would be best assessed directly on HIV-infected patient samples, the challenge of logistics due to biosafety requirements and ethics approval limit such observations. In addition, the mutation rates specifically on HIV-1 RT drug targets (i.e. IN, RT, and PR) sorely require deep understanding of the emergence of drug resistance mutations. Studying these targets specifically would be necessary, especially since sequence context and secondary structure influences the fidelity of HIV RT [74].

8. Conclusions and Future Perspective

In this review, we discussed the different cell-free and cell-based essays that contribute to the mutation rate of HIV-1, and implications in the huge range of mutation rates observed in different studies. Although numerous studies have been conducted, there is a lack of studying specific HIV-1 drug targets which is integral as sequence context and secondary structure influences the fidelity of HIV RT. Despite the full effects of mutations towards viral fitness remaining unclear, allosteric analysis have shown many distal sites in the common HIV drug targets to exhibit some allosteric effects. Together, the information may allow us to develop novel intervention strategies against this old viral foe.

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