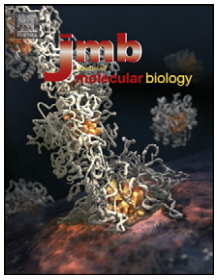

Resolution of Specific Nucleotide Mismatches by Wild-Type and AZT-Resistant Reverse Transcriptases during HIV-1 Replication

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Abstract

A key contributor to HIV-1 genetic variation is reverse transcriptase errors. Some mutations result because reverse transcriptase (RT) lacks 3′ to 5′ proofreading exonuclease and can extend mismatches. However, RT also excises terminal nucleotides to a limited extent, and this activity contributes to AZT resistance. Because HIV-1 mismatch resolution has been studied *in vitro* but only indirectly during replication, we developed a novel system to study mismatched base pair resolution during HIV-1 replication in cultured cells using vectors that force template switching at defined locations. These vectors generated mismatched reverse transcription intermediates, with proviral products diagnostic of mismatch resolution mechanisms. Outcomes for wild-type (WT) RT and an AZT-resistant (AZT^R) RT containing a thymidine analog mutation set—D67N, K70R, D215F, and K219Q—were compared. AZT^R RT did not excise terminal nucleotides more frequently than WT, and for the majority of tested mismatches, both WT and AZT^R RTs extended mismatches in more than 90% of proviruses. However, striking enzyme-specific differences were observed for one mispair, with WT RT preferentially resolving dC–rC pairs either by excising the mismatched base or switching templates prematurely, while AZT^R RT primarily misaligned the primer strand, causing deletions via dislocation mutagenesis. Overall, the results confirmed HIV-1 RT's high capacity for mismatch extension during virus replication and revealed dramatic differences in aberrant intermediate resolution repertoires between WT and AZT^R RTs on one mismatched replication intermediate. Correlating mismatch extension frequencies observed here with reported viral mutation rates suggests a complex interplay of nucleotide discrimination and mismatch extension drives HIV-1 mutagenesis.

Introduction

HIV-1 is a genetically diverse virus that persists as a quasispecies within infected individuals [1]. Many HIV-1 mutations reside in contexts characteristic of targets of host cytidine deaminases and likely result from host antiviral activities [2,3]. The possibility that host RNA polymerases also contribute to retroviral errors cannot be ruled out [4,5]. It is nonetheless clear that a significant source of genetic variation in HIV-1 populations is the mutations introduced by reverse transcriptase (RT) during processive reverse transcription or upon template switching. Current consensus suggests errors in HIV-1 genomes arise around 1 time per 50,000 bases synthesized [6]. Template switching is remarkably frequent during retroviral DNA synthesis, with RT switching from one RNA template to homologous sequences on the co-packaged viral RNA roughly 10 times during the synthesis of every proviral DNA [7–9].

Error rates based on *in vitro* experiments suggest that HIV-1 RT introduces approximately 2–5 × 10^{-4} mutations per base pair, with significantly (perhaps 10-fold) lower rates observed during replication in cultured cells [10–13]. Retroviral mutagenesis generally involves base misinsertion followed by mismatch extension, and RT's error rates are much higher than those of the cellular replication machinery. Although it lacks 3′ to 5′ proofreading exonuclease activity and frequently extends mispairs without

correction, HIV-1 RT is capable of excising incorporated bases to some extent by reversing the chemistry of polymerization in the presence of pyrophosphate or ATP [14,15]. This reaction contributes to AZT resistance, and *in vitro* reverse transcription results suggest it contributes to replication fidelity as well [16].

It is now well established that some variant RTs differ from the wild-type (WT) enzyme in nucleoside analog discrimination [17]. However, when RT mutations associated with AZT resistance in patients were first described, studies with purified enzymes failed to identify differences between RTs from AZT-sensitive and -resistant viruses, and the mechanism of HIV-1 RT's AZT resistance remained unexplained for several years [18]. It was eventually discovered that unlike many other nucleoside analog resistance-associated mutations, which act by affecting rates of nucleotide analog discrimination prior to incorporation [17], the rate of 3′ terminal nucleotide excision is significantly increased for certain AZT-resistant (AZT^R) forms of RT [19] and that this enhanced level of primer unblocking contributes to HIV-1 resistance to AZT and some other nucleoside analogs. This history of differences between experimental conditions and intracellular replication masking mechanistic properties of RT underscores the need to understand RT error mechanisms in cells as well as in purified reactions.

RT is prone to mismatch insertion in purified reactions, with some forms of RT more prone to misinsertion than others [20,21]. HIV-1 RT also corrects mismatches with a degree of selectivity: for example, G-T mismatches are rectified more frequently than C-T mismatches in purified reactions [22]. Although mutations that arise during virus replication are less well characterized mechanistically than those generated in purified reactions, differences in mutation frequency have been described for some drug resistant RT mutants during viral replication (e.g., see Refs. [23,24]) and in purified reactions. While mismatch extension occurs more frequently than nucleotide excission in purified reverse transcription reactions and some reports find no contributions of NC to fidelity [25], the addition of viral nucleocapsid protein increased the efficiency of both WT and AZT^R RT *in vitro* base excision 10-fold in another study [16]. It is possible that these and additional parameters within cells may lead to outcomes during viral replication that differ from those reported for purified reactions. Indeed, a study of error hot spots observed during viral replication demonstrated that their pattern was different from those generated on the same template in reconstituted reactions *in vitro* [12], prompting further investigation of additional differences between cells and standard *in vitro* reaction conditions that might explain differences between *in vitro* and intracellular reverse transcription outcomes [10].

Recombination, which results from template switching during reverse transcription, may also be a source of HIV-1 mutations. When RT reaches the end of a template, it can add non-templated nucleotides [26,27]. Upon template switching *in vitro*, extension of non-templated bases leads to mutations at the point of strand transfer in up to 30–50% of all reverse transcription products [28–30]. Recombination in cells appears to be much less error prone [27,28,30,31], but template switch-associated mutagenesis has been reported, with some reports suggesting that up to 20% of RT mutations may be associated with template switching [32–34]. However, the fact that recombinogenic template switching can occur at many, if not all, template positions in viral replication products complicates addressing whether or not mutations observed within crossover intervals arose upon template switching or by a different mechanism.

Although mismatch extension and excision by HIV-1 RT have been studied in purified reactions *in vitro*, there is currently no data directly addressing these processes in a cell-based system. In the current study, we describe a novel system for studying HIV-1 RT mismatch resolution during single rounds of replication in human cells. These studies use retroviral inside-out (RIO) vectors, which were designed to promote template switching at defined template positions during reverse transcription. The work here demonstrates that RIO vectors were packaged efficiently and were able to successfully complete single rounds of reverse transcription and integration when mobilized by helpers harboring either WT or an AZT^R RT variant [19]. Additionally, forced copy-choice recombination occurred as intended at the donor template's 5′ terminus. Using this system, specific mismatches were introduced and three independent approaches were used to determine the mechanisms by which HIV-1 WT and AZT^R RTs resolved these. The results indicated that HIV-1 RT displays nucleotide-specific differences in mismatch extension during virus replication and that the spectra of mechanisms used for the resolution of at least one mismatch differ between WT and AZT^R RTs.

Results

Establishing vectors to monitor mismatch resolution during HIV-1 replication

In this study, the mechanisms of primer-terminal mismatch resolution during HIV-1 replication were examined using novel vectors designed to promote template switching at defined template positions in cultured cells. These vectors, which are called RIO vectors, contain virus-derived sequences in a circularly permuted order (Fig. 1a). These vectors were designed to circumvent one challenge to