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Review

Viral reverse transcriptases

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ABSTRACT

Reverse transcriptases (RTs) play a major role in the replication of *Retroviridae*, *Metaviridae*, *Pseudoviridae*, *Hepadnaviridae* and *Caulimoviridae*. RTs are enzymes that are able to synthesize DNA using RNA or DNA as templates (DNA polymerase activity), and degrade RNA when forming RNA/DNA hybrids (ribonuclease H activity). In retroviruses and LTR retrotransposons (*Metaviridae* and *Pseudoviridae*), the coordinated action of both enzymatic activities converts single-stranded RNA into a double-stranded DNA that is flanked by identical sequences known as long terminal repeats (LTRs). RTs of retroviruses and LTR retrotransposons are active as monomers (e.g. murine leukemia virus RT), homodimers (e.g. Ty3 RT) or heterodimers (e.g. human immunodeficiency virus type 1 (HIV-1) RT). RTs lack proofreading activity and display high intrinsic error rates. Besides, high recombination rates observed in retroviruses are promoted by poor processivity that causes template switching, a hallmark of reverse transcription. HIV-1 RT inhibitors acting on its polymerase activity constitute the backbone of current antiretroviral therapies, although novel drugs, including ribonuclease H inhibitors, are still necessary to fight HIV infections. In *Hepadnaviridae* and *Caulimoviridae,* reverse transcription leads to the formation of nicked circular DNAs that will be converted into episomal DNA in the host cell nucleus. Structural and biochemical information on their polymerases is limited, although several drugs inhibiting HIV-1 RT are known to be effective against the human hepatitis B virus polymerase. In this review, we summarize current knowledge on reverse transcription in the five virus families and discuss available biochemical and structural information on RTs, including their biosynthesis, enzymatic activities, and potential inhibition.

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Abbreviations: AIDS, acquired immune deficiency syndrome; ALV, avian leukosis virus; AMV, avian myeloblastosis virus; APOBEC, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like; ASLV, avian sarcoma leukosis virus; AZT, 3′-azido-3′-deoxythymidine; BIV, bovine immunodeficiency virus; BLV, bovine leukemia virus; CA, capsid protein; CaMV, cauliflower mosaic virus; cccDNA, covalently closed circular DNA; cDNA, complementary DNA; cPPT, central polypurine tract; CRISPR, clustered regularly interspaced short palindromic repeats; DHBNH, dihydroxy benzoyl naphthyl hydrazine; DIS, dimerization initiation site; DR, direct repeat; dsDNA, double-stranded DNA; dslDNA, double-stranded linear DNA; EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; EIAV, equine infectious anemia virus; FIV, feline immunodeficiency virus; FLV, feline leukemia virus; GPD, G-patch domain; HAART, highly active antiretroviral therapy; HBV, hepatitis B virus; HIV-1, human immunodeficiency virus type 1; HIV-2, HIV type 2; HTLV-I, human T-cell leukemia virus type I; HTLV-II, HTLV type II; IN, integrase; LTR, long terminal repeat; MA, matrix protein; MLV, murine leukemia virus; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; mRNA, messenger RNA; msDNA, multicopy single-stranded DNA; NC, nucleocapsid protein; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PBMCs, peripheral blood mononuclear cells; PBS, primer binding site; PDB, Protein Data Bank; pgRNA, pregenomic RNA; PPT, polypurine tract; PR, protease; R, repeat; rcDNA, relaxed circular DNA; RNase H, ribonuclease H; RSV, Rous sarcoma virus; RT, reverse transcriptase; SFV, simian foamy virus; SIV, simian immunodeficiency virus; (+)ssDNA, plus-strand strong-stop DNA; (−)ssDNA, minus-strand strong-stop DNA; TAMs, thymidine analogue resistance mutations; TP, terminal protein; tRNA, transfer RNA; U3, unique 3′; U5, unique 5′; XMRV, xenotropic murine leukemia virus-related virus.

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1. Introduction

The discovery of retroviral reverse transcriptases (RTs) in 1970 represented a major breakthrough with an enormous impact on life sciences (Baltimore, 1970; Temin and Mizutani, 1970; for a historical account, see Coffin and Fan, 2016). These enzymes were described as nucleic acid polymerases able to synthesize a complementary DNA (cDNA) using RNA as template. Their discovery challenged the prevailing central dogma of molecular biology, which states that DNA is transcribed into RNA, which is then translated into proteins. Further studies showed that RTs were able to use RNA or DNA as templates and had ribonuclease H (RNase H) activity. The RNase H activity of the RTs facilitates cleavage of the RNA strand when forming part of RNA/DNA heteroduplexes. Reverse transcription (i.e. the conversion of RNA to DNA by an RT) plays an important role in the replication of *Retroviridae*, *Metaviridae*, *Pseudoviridae*, *Hepadnaviridae* and *Caulimoviridae*. RTs encoded in their genomes are phylogenetically related to those found in mobile genetic elements of prokaryotes and eukaryotes (Fig. 1). Amino acid sequence alignments of RT DNA polymerase domains of different clades show the conservation of a series of motifs (designated as 1, 2, A, B, C, D and E), required for enzymatic function (Fig. 2).

The role of RTs in the replication of retroviruses (then known as 'RNA tumor viruses') gave them notorious relevance for understanding malignant transformation. However, massive genome DNA sequencing has revealed that eukaryotes can have more RT genes than genes encoded for any other protein (Dewannieux and Heidmann, 2013). Most of them are retrotransposable elements or retrotransposons, which were initially discovered in model organisms such as yeast (i.e. *Saccharomyces cerevisiae*) and the fruitfly (*Drosophila melanogaster*) (Eickbush and Jamburuthugoda, 2008). In addition to its presence in mobile genetic elements in plants and animals, RT activity is associated with the replication of chromosome ends (telomerase). In prokaryotes, RTs have been found in the coding region of an extrachromosomal satellite DNA, known as multicopy single-stranded DNA (msDNA) (Inouye et al., 1990; Lampson et al., 2005) and in mobile genetic elements known as group II introns that contain an N-terminal RT (RNA-dependent DNA polymerase) domain followed by an RNA-binding maturase domain (Lambowitz and Zimmerly, 2004) (Fig. 1).

Retroviridae, *Metaviridae* and *Pseudoviridae* share similar mechanisms of replication and form double-stranded DNA (dsDNA) that integrates into the host cell genomes. The integrated form (proviral DNA) contains direct sequence repeats that flank the internal coding region and are known as long terminal repeats (LTRs). All retroviruses have three major genes, arranged in the order 5′-*gag*-*pol*-*env*-3′ and can be transmitted horizontally from cell to cell. The *gag* gene encodes structural proteins including those forming the viral capsid, the *pol* gene encodes enzymatic activities needed for virus propagation (i.e. protease (PR), RT and integrase (IN)), and *env* contains the genetic information needed for expression of proteins mediating infection and viral entry.

Metaviridae and *Pseudoviridae* are LTR retrotransposons found in most eukaryotes. Their genomic structure includes the *gag* and *pol* genes that can be organized and expressed in many different ways (Havecker et al., 2004; Curcio et al., 2015). LTR retrotransposons form virus-like particles but unlike retroviruses, most of them can only be transmitted from site to site within the genome of a single cell. However, a number of LTR retrotransposons encode an envelope protein, which allows the virus particle to infect another cell. The best characterized examples of *env*-containing retroelements are the *Drosophila* errantiviruses (*Metaviridae*) *gypsy* and *ZAM*. The *gypsy* element has been shown to be infectious and can be transmitted from one individual to another (Leblanc et al., 2000). Genomic studies have shown the widespread distribution of *env*-like genes in *Pseudoviridae* (sireviruses) and *Metaviridae* (errantiviruses, metaviruses and semotiviruses) (Malik et al., 2000; Eickbush and Malik, 2002).

The pararetroviruses, found in both plants (*Caulimoviridae* family) and animals (*Hepadnaviridae* family) have rather different replication cycles and replication mechanisms. Hepadnaviruses are small enveloped DNA viruses that infect birds and mammals. Hepatitis B virus (HBV) is the prototypic member of this family and one of the most dangerous human pathogens. Their genome is a partially double-stranded relaxed circular DNA (rcDNA) molecule of about 3 kb that is transported into the nucleus where it is converted into a covalently closed circular DNA (cccDNA). This episomal DNA is used as template for cellular RNA polymerase II to produce various RNAs, including a pregenomic RNA (pgRNA). After encapsidation with core protein and the viral RT, the pgRNA is converted to rcDNA, while rendering mature viral nucleocapsids (Lucifora and Zoulim, 2011; Hu and Seeger, 2015). The hepadnavirus RT DNA polymerase activity could participate in the synthesis of cccDNA from rcDNA, although its specific role is still unknown. In all hepadnaviruses, double-stranded linear DNA (dslDNA) is also synthesized as a minor viral DNA species during reverse transcription. This DNA is infectious, and its circularization by non-homologous recombination leads to the formation of cccDNA (Yang and Summers, 1998), although this process is not accurate and cannot sustain replication.

Caulimoviridae are viruses transmitted by aphids that infect dicotyledonous plants and replicate by reverse transcription of a pregenomic RNA. The genome of caulimoviruses consists of a double-stranded circular DNA in an open form due to the presence of sequence discontinuities that originate during the reverse transcription process (Haas et al., 2002). The replication of caulimoviruses resembles that of *Hepadnaviridae* with the viral DNA forming supercoiled mini-chromosome structures upon entering the host nucleus. Unlike retroviruses but similar to hepadnaviruses, integration into the host genome is not required for

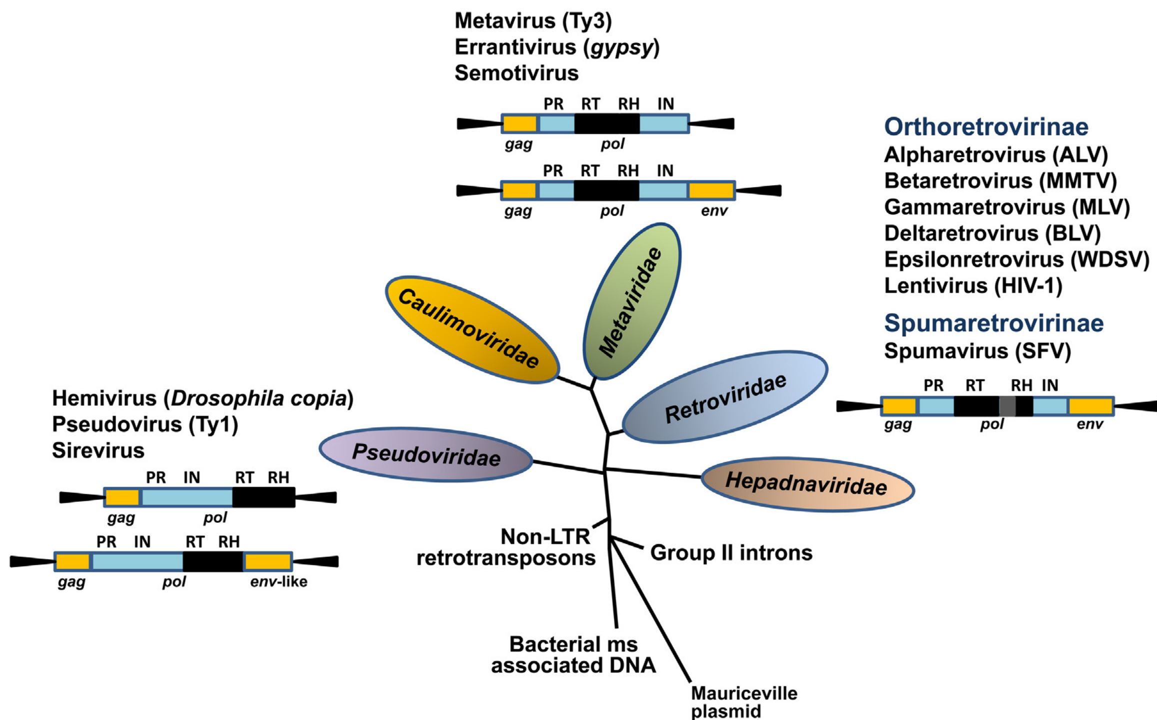

Fig. 1. Phylogenetic tree based on the sequences of RTs from different clades. The colored ovals represent RTs of viruses from the same family. Genome organizations showing relevant genes and the location of the protease (PR)-, reverse transcriptase (RT)-, RNase H (RH)-, and integrase (IN)-coding regions are shown for *Retroviridae*, *Metaviridae* and *Pseudoviridae*. Subfamilies and/or genera belonging to each family are shown above the corresponding genomes. Prototypic species are shown between parentheses. Abbreviations are: ALV, avian leukosis virus; MMTV, mouse mammary tumor virus; MLV, murine leukemia virus; BLV, bovine leukemia virus; WDSV, Walleye dermal sarcoma virus; HIV-1, human immunodeficiency virus type 1; SFV, simian foamy virus. The tree was obtained with >100 RT sequences and based on one published by the International Committee on Taxonomy of Viruses (King et al., 2012).

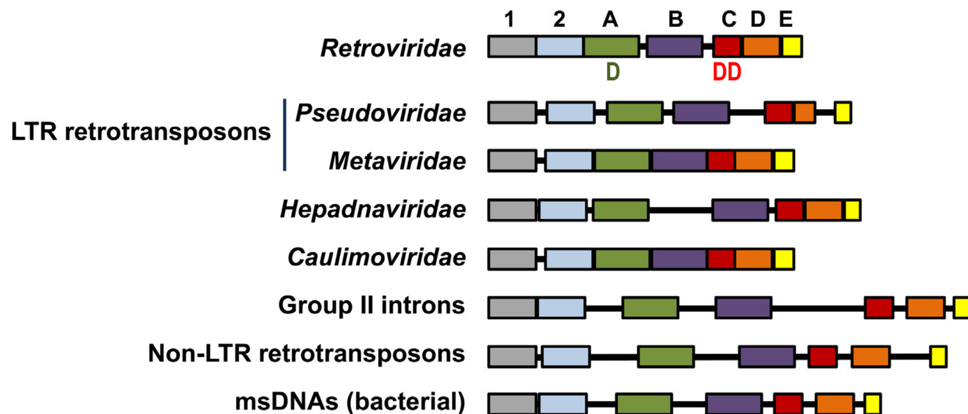

Fig. 2. Conserved sequence motifs found in the DNA polymerase domains of RTs. Location of conserved motifs (defined by Xiong and Eickbush (1990)) are indicated by colored boxes. Catalytic aspartic residues (D) are indicated in motifs A and C (Poch et al., 1989).

caulimovirus replication, and their genome lacks an integrase-coding region (Hohn and Rothnie, 2013). True retroviruses are not present in plants. However, endogenous pararetroviral sequences derived from *Caulimoviridae* have been found in the genomes of many Angiosperms (Chabannes and Iskra-Caruana, 2013). The *Caulimoviridae* family is composed of six genera (*Caulimovirus*, *Petuvirus*, *Soymovirus*, *Cavemovirus*, *Badnavirus* and *Tungrovirus*) and the most relevant representative is the cauliflower mosaic virus. There is little information on the structure of their RT but it is believed that in the cauliflower mosaic virus the enzyme originates from a polyprotein homologous to the *pol* gene product of retroviruses, with PR, DNA polymerase and RNase H domains.

Despite the important similarities between RTs of different viruses, major research efforts have been devoted to understanding the mechanism of reverse transcription in retroviruses, and the structure of human immunodeficiency virus type 1 (HIV-1) RT as a target of antiretroviral therapy (Menéndez-Arias, 2013a). Although there is a vast literature on those topics, in this review we will provide a broader update covering the most widely studied retroviral RTs and their homologous enzymes in LTR retrotransposons, hepadnaviruses and caulimoviruses.

2. Reverse transcription in retroviruses and LTR retrotransposons

In retroviruses and LTR retrotransposons, RT converts ssRNA into dsDNA. The DNA polymerase and RNase H activities of the RT are sufficient to complete all of the steps in the process, although

the viral nucleocapsid (NC) protein is a necessary partner (for reviews see Thomas and Gorelick, 2008; Rein, 2010; Darlix et al., 2011). The mature NC protein (p7) has a chaperoning role in reverse transcription facilitating strand transfer events. In addition, NC seems to control early viral DNA synthesis during virus assembly and budding, although the precise mechanism is unknown. *In vitro*, NC increases RT processivity and fidelity of DNA synthesis (Kim et al., 2012), although these effects have not been reported *in vivo*.

The genomic viral ssRNA is similar to a messenger RNA (mRNA) and contains a 5′ cap and a polyadenylated tail. The dsDNA is flanked by LTRs. In HIV-1 those sequences have around 634 base pairs, and like in other retroviruses and LTR retrotransposons, they are segmented into three regions, known as U3 (unique 3′), R (repeat) and U5 (unique 5′). The LTR ends participate in the integration of the dsDNA into the host genome.

The reverse transcription process is outlined in Fig. 3. Reverse transcription initiates after binding of a cellular transfer RNA (tRNA) to the primer binding site (PBS) (reviewed in Abbink and Berkhout, 2007). The PBS is a structured RNA element located close to the 5′ end of the RNA genome in the untranslated leader region. The 3′-OH of the tRNA serves as a primer for RNA-dependent DNA synthesis. Retroviruses use different tRNA primers (Marquet et al., 1995; Mak and Kleiman, 1997). All lentiviruses (including HIV-1 and HIV type 2 (HIV-2)) and the mouse mammary tumor virus (MMTV) use $tRNA^{Lys3}$. Avian retroviruses (e.g. Rous sarcoma virus (RSV)) use $tRNA^{Trp}$, many mammalian retroviruses (e.g. human T-cell leukemia viruses types 1 and 2 (HTLV-I and HTLV-II, respectively) and murine leukemia virus (MLV)) use $tRNA^{Pro}$, and spumaviruses use $tRNA^{Lys1,2}$. Studies with HIV-1 mutants carrying altered PBS sequences corresponding to other tRNA species ($tRNA^{Ile}$, $tRNA^{Lys1,2}$, $tRNA^{Phe}$, $tRNA^{Pro}$, $tRNA^{Trp}$) demonstrated that they were able to replicate, but at a smaller rate than the wild-type HIV-1 (Das et al., 1995; Abbink and Berkhout, 2007). Priming by tRNAs is also observed in LTR retrotransposons. Thus, the metavirus Ty3 as well as the pseudoviruses Ty1, Ty5 and copia use $tRNA_i^{Met}$ (Chapman et al., 1992; Keeney et al., 1995; Ke et al., 1999). An exception to this rule was found in the metavirus Tf1 of *Schizosaccharomyces pombe*. In these elements, the first 11 bases of the primary RNA transcript anneal to a sequence downstream of the 5′ LTR at the typical tRNA PBS. The annealed transcript is excised by the RNase H activity of the Tf1 RT and then used as primer in reverse transcription (Lin and Levin, 1997).

In HIV-1, the 3′-terminal 18 nucleotides of the tRNA primer base pair with the complementary PBS (positions +182 to +199) in the viral genome. Studies with the HIV-1 RT have shown that the incorporation of the first 5–6 nucleotides at the 3′ end of the tRNA primer is slow and difficult, and then the process speeds up considerably (Isel et al., 1996). The tRNA is extended up to the 5′ end of the genome, generating an intermediate known as minus-strand strong-stop DNA ((−)ssDNA). RNase H cleavage removes the 5′ end of the viral RNA and exposes the newly synthesized (−)ssDNA. The viral RNA contains duplicated sequences at both ends, known as repeats (R). The R sequence in the (−)ssDNA facilitates the strand transfer event by which DNA synthesis can continue from the 3′ of the viral RNA. Retroviral minus-strand DNA synthesis can resume on the same RNA or on a different RNA template (Panganiban and Fiore, 1988; Hu and Temin, 1990a; Wilhelm et al., 1999). In the proviral DNA of HIV-1, a conserved tract of three guanosines is located at the U3/R junction. HIV-1 transcripts containing one, two or three guanosines at their 5′ end are expressed in infected cells, and have been detected in the virion. It has been shown that the number of guanosines at the 5′ end of the viral genomic RNA is a major determinant for successful strand transfer of the (−)ssDNA (Masuda et al., 2015).

The elongation of the (−)ssDNA continues with simultaneous degradation of the template RNA. However, all retroviruses have a purine-rich sequence in the RNA genome that resists cleavage by the RNase H activity of the RT. This polypurine tract (known as PPT) is located at the U3 region. In HIV-1 and other retroviruses, there is an additional PPT located in the central part of the genome (cPPT). These polypurine tracts serve as primers for synthesis of plus-strand DNA. The cPPT does not seem to be essential for virus replication but increases the efficiency of the process (Charneau et al., 1992; Hungnes et al., 1992). Recently published work suggests that the G-rich sequence of the cPPT could play an important role in keeping the RNA genomes in close proximity, thereby facilitating recombination in numerous hot spots (Piekna-Przybylska et al., 2013).

After addition of a few nucleotides, the PPTs are removed from the plus-strand DNA by the RNase H activity of the RT. Plus-strand DNA synthesis originating from the 3′-PPT continues to the 5′-end of the minus-strand DNA until it reaches the 18th nucleotide in the tRNA where further synthesis is blocked due to the presence of a methylated base (Swanstrom et al., 1981). The obtained DNA is designated as plus-strand strong stop DNA ((+)ssDNA). The tRNA is then removed by RT's RNase H activity. In most retroviruses, cleavage occurs at the 3′ end of the tRNA, although in the case of HIV-1, the completed minus-strand DNA contains adenosine monophosphate (riboA) at its 5′end (Whitcomb et al., 1990; Pullen et al., 1992; Smith and Roth, 1992) (Fig. 3).

The second strand transfer reaction involves the annealing of the (+)ssDNA to the 3′ end of the full-length minus-strand DNA, through base pairing of the complementary PBS sequences (Yu et al., 1998). Then, the strand displacement activity of the retroviral RT facilitates the completion of the process and the formation of a full-length, integration-competent, double-stranded DNA with two identical LTRs, one at each end. The strand displacement activity of the RT can also generate flaps constituted by three-stranded structures with overlapping positive-strand sequences (Fig. 3). These flaps are abundant in avian sarcoma leukosis virus (ASLV), where plus-strand DNA synthesis is initiated at many sites. In contrast, MLV has only one initiation site that corresponds to the 3′ PPT. The presence of cPPTs in HIV-1 or the retrotransposon Ty1 facilitates the formation of DNA flaps of 88–130 nucleotides. The functional role of these flaps is still uncertain. Cellular endonucleases and ligases can remove the flaps and join the DNA ends to generate a complete provirus that can eventually integrate in the host cell genome.

There is a vast literature on the effects of viral proteins and cell factors on reverse transcription. In most cases, studies have been carried out in the context of HIV-1 infections. However, their mechanism of action and significance is not always clear. It is assumed that reverse transcription initiates in the cytoplasm of infected cells, within a structure known as reverse transcription complex that contains RT and other viral proteins (for a detailed discussion, see Hughes, 2015). The capsid protein (CA) of HIV-1 forms a shell where reverse transcription initiates although a remodeling process may occur while the viral ssRNA is converted to dsDNA. However, other viral proteins such as Nef, Tat, Vif, Vpr, the matrix protein (MA) and the integrase may influence this process (Tekeste et al., 2015). Retroviral dUTPases are also known to influence replication in several retroviruses (e.g. feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV) or MMTV) (Elder et al., 1992; Köppe et al., 1994; reviewed in Hizi and Herzig, 2015). In addition, cellular proteins such as cyclophilin A, topoisomerase I, cytidine deaminases (apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) family), SAMHD1 and the eukaryotic elongation factor 1 (eEF1) have been identified as potential cofactors that affect directly or indirectly HIV-1 reverse transcription (reviewed in Henriet et al., 2009; Warren et al., 2009). A systematic analysis of the available evidence is beyond the scope of a general introduction such as the one intended in this paper, and

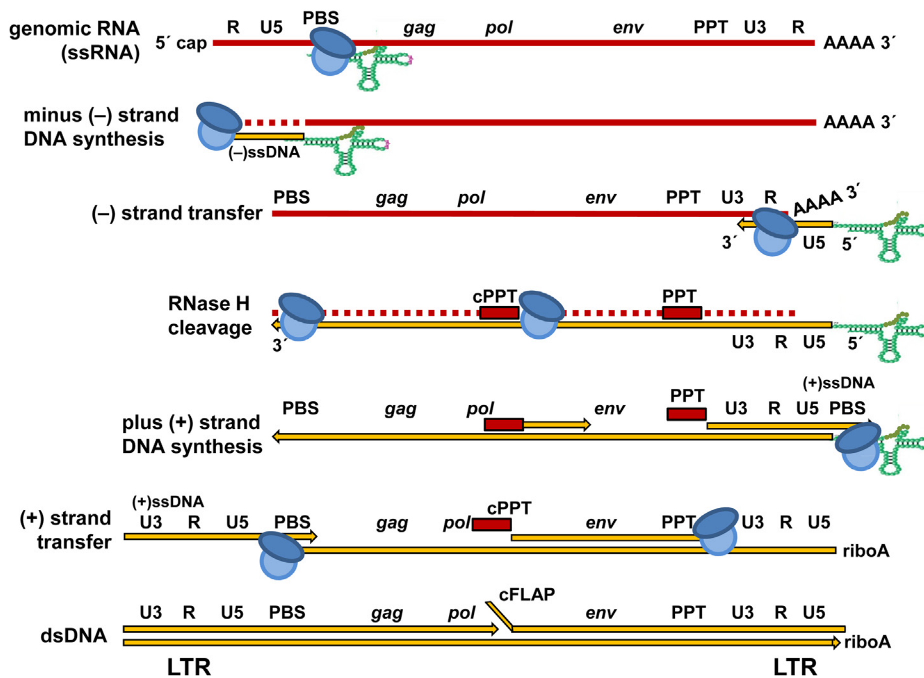

Fig. 3. HIV-1 reverse transcription. The steps involving the conversion of single-stranded genomic RNA into double-stranded DNA are shown. RNA is shown in red and DNA in orange. The heterodimeric RT subunits are shown as dark and light blue ovals.

interested readers may find relevant information in a few recently published reviews (Harris et al., 2012; Hilditch and Towers, 2014; Fujiwara, 2015; Hughes, 2015).

3. RT biosynthesis in retroviruses and LTR retrotransposons

In retroviruses and LTR retrotransposons, RTs are encoded within the *pol* gene. In most retroviruses, this gene contains coding sequences for the viral PR, RT (DNA polymerase plus RNase H domains) and IN proteins. In some retroviruses (e.g. MMTV and other betaretroviruses), the PR-coding region appears in a separate open reading frame known as *pro*. RT is normally expressed as part of a large precursor known as Gag-Pol or Gag-Pro-Pol that includes structural proteins such as MA (matrix), CA (capsid) and NC (nucleocapsid). The structural proteins are encoded within the *gag* gene. However, suppression of the stop codon at the end of *gag* facilitates the synthesis of large precursors. The stop codon can be bypassed by read-through suppression or −1 frameshifting (Jacks et al., 1987). The first mechanism occurs when *gag* and *pol* are in the same open reading frame and separated by a stop codon that is read as Gln with a 5% frequency. This mechanism is characteristic of gammaretroviruses (e.g. MLV). In contrast, ribosomal frameshifting occurs in retroviruses where *gag* and *pro*, *gag* and *pol* and/or *pro* and *pol* are in a −1 reading frame (Fig. 4). When the *pol* gene contains the PR-coding region (e.g. in HIV-1), this mechanism allows after only one frameshift event, the synthesis of equimolecular amounts of PR, RT and IN, which are produced in a 20-fold reduced amount relative to structural proteins encoded within the *gag* gene. In viruses having *gag*, *pro* and *pol* in different open reading frames (e.g. MMTV or HTLV-I), RT and IN derive from a Gag-Pro-Pol precursor, originated after two frameshift events (Jacks et al., 1987; Hatfield and Oroszlan, 1990) (Fig. 4).

Spumaviruses (foamy viruses) are unique retroviruses because they do not produce Gag-Pol or Gag-Pro-Pol polyproteins and the RT is generated from a separate spliced mRNA (Rethwilm, 2003; Rethwilm, 2010). This RNA lacks the *gag* sequence and guides the synthesis of the Pol polyprotein that contains the enzymes PR, RT and IN. Another interesting feature of spumaviruses (also shown by hepadnaviruses) is that infectious particles can contain dsDNA (as well as single-stranded RNA), suggesting that reverse transcription occurs during polyprotein packaging and maturation before the virion enters the target cells (Linial and Eastman, 2003; Lee et al., 2013).

The subunit composition of retroviral RTs is rather diverse (Fig. 4). Three enzymatically-active RT isoforms have been purified from virions of alpharetroviruses, such as avian leukosis virus (ALV), ASLV, avian myeloblastosis virus (AMV) or RSV. The most abundant isoform in those viruses is the α/β heterodimer. It contains DNA polymerase, RNase H and IN domains in the β subunit (95 kDa) and DNA polymerase and RNase H domains in the α chain (63 kDa). Other isoforms found in purified virions are homodimers (β/β) and monomers (α) (Hizi and Joklik, 1977). Proteolytic cleavage of the Gag-Pol precursor to obtain β/β homodimers is needed to obtain an active RT. Then, further processing by the viral PR leads to the formation of α/β heterodimers that can dissociate to monomers α and β, although only the β subunit retains activity (for a detailed review, see Herschhorn and Hizi, 2010).

Active heterodimeric RTs have also been found in lentiviruses. However, their size is smaller. HIV-1 virions contain RTs made of 66 and 51 kDa subunits, designated as p66 and p51, respectively. The larger subunit has 560 residues and contains DNA polymerase and RNase H domains, while p51 (440 residues) lacks the RNase H domain. The p51 subunit has a major role in the stabilization of the heterodimer. In addition, p51 contributes to tRNA binding during initiation of reverse transcription (Arts et al., 1996) and accommodates the RNA/DNA substrate for correct positioning in the RNase H active site (Chung et al., 2013). Although the proteolytic events leading to the formation of p66/p51 *in vivo* have not been demonstrated, p66 homodimers can be converted to p66/p51 *in vitro* by the action of the viral PR. Similar heterodimeric compositions have been described for RTs of HIV-2 (p68/p55), simian immunodeficiency virus (SIV) (p66/p51), FIV (p67/p54) and bovine immunodeficiency virus (BIV) (p64/p51) (Hizi and Herschhorn, 2008). Biochemical studies have shown that HIV-1 RT undergoes a complex maturation process that includes three basic steps: (i) domain rearrangements; (ii) dimerization; and (iii) subunit-

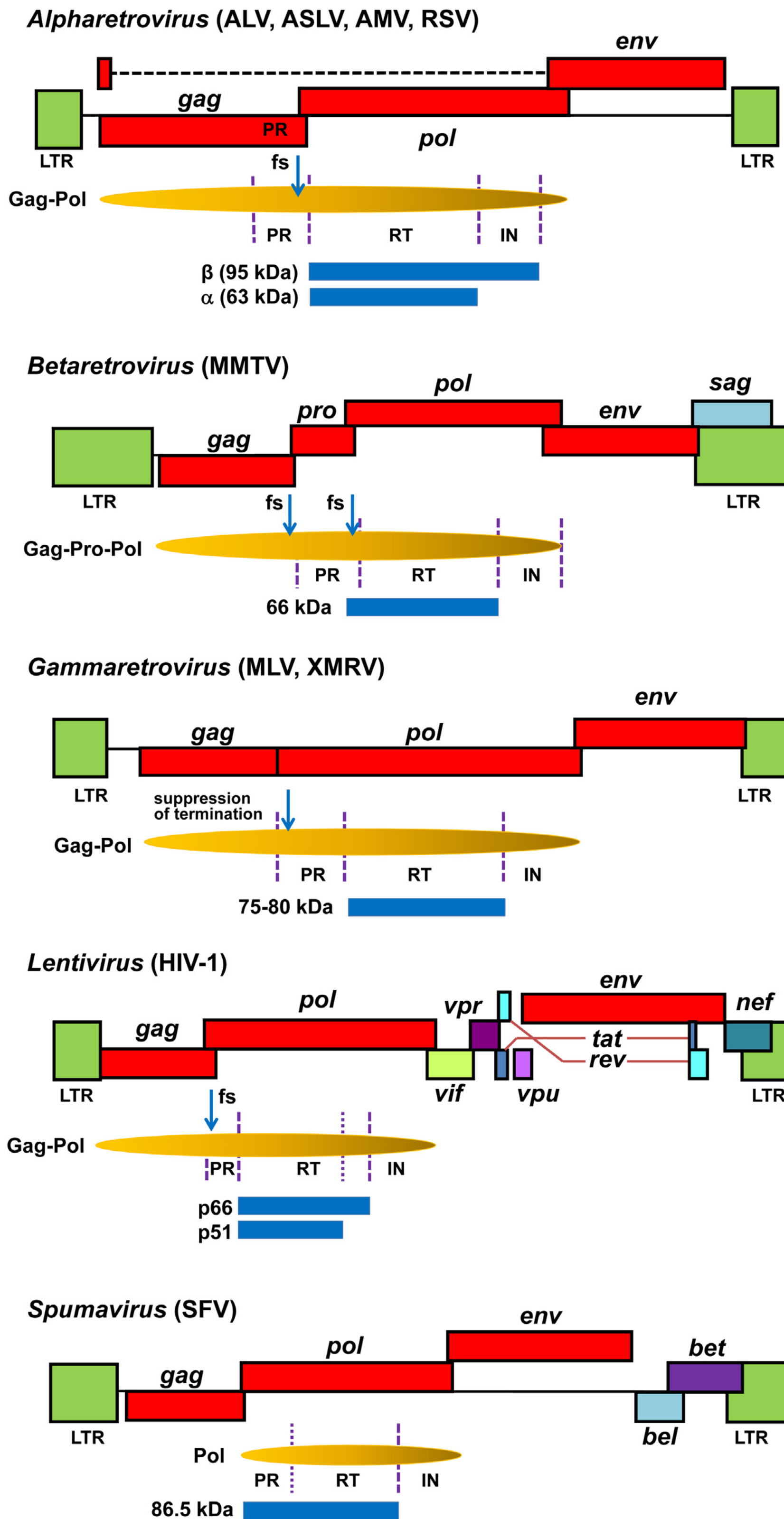

Fig. 4. RT biosynthesis in selected retroviruses. The figure shows the genetic organizations of prototypic retroviruses and the location of LTRs (green boxes) and major genes (*gag*, *pol* and *env*) (red boxes). The RT subunits (shown in dark blue) derive from cleavage of Gag-Pol, Gag-Pro-Pol and Pol precursors (orange ovals). Vertical discontinuous lines indicate cleavage sites at the N and C terminal ends of the PR, RT and IN. Frameshift (fs) positions and the site of suppression of a codon stop are indicated with vertical arrows. The size of the retroviral genomes shown range from 7.6 to 11.6 kb, but for the sake of clarity, all were normalized to the same size.

selective RNase H proteolysis (for a recent review, see London, 2016). Amino acid changes that destabilize the heterodimeric structure of the RT could have a negative effect on polyprotein processing (Abram and Parniak, 2005; Wapling et al., 2005; Olivares et al., 2007; Dunn et al., 2009). In some cases, the deleterious effect on viral fitness can be compensated by mutations in the viral PR that

modulate the kinetics of the proteolytic process (Olivares et al., 2007).

In contrast, RTs of gammaretroviruses such as MLV, feline leukemia virus (FLV) or the xenotropic MLV-related virus (XMRV) are active monomers of 75–80 kDa that contain a DNA polymerase domain plus an RNase H domain (Roth et al., 1985; Misra et al., 1998; Operario et al., 2005; Barrioluengo et al., 2012; Ndongwe et al., 2012). Monomers or homodimers of less-studied RTs have been obtained in active forms using recombinant DNA technology. However, their subunit composition in the virion remains unknown. For example, the MMTV RT has been obtained as a 66-kDa polypeptide of 630 amino acids, containing 27 residues at its N-terminal end that derived from the C-terminal region of the viral PR (Taube et al., 1998). Using specific antisera, researchers have also shown evidence of the presence in virions of mature MMTV RT fused to PR-derived sequences (Entin-Meer et al., 2003). On the other hand, mutagenesis studies have shown that an N-terminal extension including a glycine-rich region known as the G-patch domain (GPD) is required for optimal Mason-Pfizer monkey virus (MPMV) RT activity (Křízová et al., 2012). The GPD sequence is found in several betaretroviruses but not in MMTV, and is located between the PR- and the RT-coding regions of their genome. Released MPMV virions contain PR variants with and without the GPD sequence, which have similar proteolytic activities and substrate specificities (Zábranský et al., 1998). Based on immunological evidence, Křízová et al. proposed that the MPMV RT is a heterodimer with a small subunit that contains the GPD sequence.

As observed with the MMTV RT and suggested for the MPMV polymerase, the bovine leukemia virus (BLV) RT was also expressed and purified with an N-terminal extension. This enzyme was active as a monomer of 584 amino acids that contained 26 residues derived from the viral PR (Perach and Hizi, 1999). Despite being also a deltaretrovirus, the most likely active form of the HTLV-I RT is a p62/p49 heterodimer similar to those found in lentiviruses. Nevertheless, a precursor of 98 kDa containing the RT fused to the IN has been detected in HTLV-I virions (Mitchell et al., 2006). Expression and biochemical characterization of recombinant HTLV-I RT has not been yet reported.

In spumaviruses, the polyprotein Pol is cleaved between the RNase H and the IN domains to produce a mature PR-RT and the IN (Fig. 4). The PR-RT polypeptide found in mature virions is monomeric (Hartl et al., 2008), although dimerization of the PR is required for proteolytic activity (Hartl et al., 2011). Viral PR-mediated cleavage of Gag is required for initiation of reverse transcription, although the unprocessed Pol precursor has RT and IN activities within the viral capsid (Hütter et al., 2013; Spannaus et al., 2013). The PR domain corresponds to the first 101 amino acids of Pol, while residues 102–751 include the DNA polymerase and RNase H domains of the RT. Using *pol* deletion mutants of a simian foamy virus (SFV) infecting macaques, researchers have shown that the sequence comprising residues 107–143 is critical for polymerase activity but not for protease activity. In addition, the connection subdomain (residues 454–590) modulates RT function, while affecting substrate affinity and protein stability (Schneider et al., 2014). In contrast to orthoretroviruses, reverse transcription is a late event in foamy virus replication that takes place in producer cells. Evidence indicates that this can be attributed to the Pol polyprotein itself, because the expression of the spumavirus RT as an engineered Gag-Pol polyprotein did not affect the timing of reverse transcription (Jackson et al., 2013).

A number of RTs derived from LTR retrotransposons have been purified and characterized. Examples are the *Saccharomyces cerevisiae* Ty1 and Ty3 RTs and the *Saccharomyces pombe* Tf1 RT. These enzymes were expressed and purified in *E. coli* as single polypeptides of around 55–65 kDa, with histidine tails at their C-terminal ends (Rausch et al., 2000; Wilhelm et al., 2000; Kirshenboim et al., 2007). Successful expression of Ty1 RT required the addition of a small acidic tail at the N-terminus of the polymerase domain that mimics the C-terminal region of the Ty1 IN (Wilhelm et al., 2000; Wilhelm and Wilhelm, 2005). Structural studies have recently shown that the Ty3 RT adopts an asymmetric homodimeric architecture whose assembly is substrate dependent (Nowak et al., 2014).

4. RTs of retroviruses and LTR retrotransposons

4.1. Structural studies

Current knowledge on the mechanism of action of retroviral RTs is based on many available crystal structures of HIV-1 RTs (for reviews, see Ren and Stammers, 2008; Sarafianos et al., 2009). Among them, the ternary complex of HIV-1_{HXB2} RT with dsDNA and dTTP (PDB file 1RTD; Huang et al., 1998) is probably one of the most relevant to understand the influence of specific amino acids on DNA polymerase and RNase H activities (Fig. 5). Other structures that provide additional insights into RT function are: (i) binary complexes of RT bound to dsDNA (PDB files 3KJV, 3KK1, 3KK2, 3KK3, 1T05 and 2HMI) (Ding et al., 1998), (ii) HIV-1 RTs in complex with dsDNA in pre- and post-translocation states (1N5Y, 1N6Q) (Sarafianos et al., 2002), (iii) HIV-1 RT bound to a cleavage-resistant polypurine tract (1HYS) (Sarafianos et al., 2001), and (iv) other structures including the apoenzyme alone, bound to RT inhibitors or complexed with nucleic acids with or without antiretroviral drugs (e.g. 4B3O, 4B3P, 4B3Q, 4PUO, 4PWD, 4Q0B, 3JYT, 3JSM, etc.) (Tuske et al., 2004; Ren and Stammers, 2008; Das et al., 2009; Tu et al., 2010; Das et al., 2012; Lapkouski et al., 2013).

Unlike in the case of HIV-1 RT, there is only one crystal structure available for HIV-2 RT (Ren et al., 2002). It is an unliganded form of the enzyme with subunits of 559 and 427 residues, respectively. Although the size of the small HIV-2 RT subunit *in vivo* is uncertain, biochemical evidence suggests that cleavage leading to the formation of p55 occurs after Met^{484}, and not at the equivalent site of HIV-1 RT (i.e. between Phe^{440} and Tyr^{441}) (Fan et al., 1995). The smaller size of this subunit in the crystal structure has been attributed to degradation of p68 by bacterial proteases (Bird et al., 2003).

Crystal structures of HIV-1 and HIV-2 RTs show similar folds. In HIV RTs both subunits contain four subdomains, designated fingers, palm, thumb and connection, whereas the large subunit has a C-terminal RNase H domain. Despite their sequence identity, the spatial organization of the subdomains differs substantially in both subunits. The p51 subunit is tightly packed and cannot participate in the catalysis. On the other hand, p66 adopts an open conformation and provides the catalytic residues for the DNA polymerase (Asp^{110}, Asp^{185} and Asp^{186}) and RNase H (Asp^{443}, Glu^{478}, Asp^{498} and Asp^{549}) activities, as well as most of the nucleic acid binding cleft interactions (Fig. 5). The fingers, palm and thumb subdomains of p66 provide lateral and apical interactions with the nucleic acid substrate, while the 'floor' of the cleft is provided by the connection subdomains of both subunits and the thumb subdomain of p51 (Jacobo-Molina et al., 1993). The nucleic acid binding cleft can accommodate 17–18 base pairs between the active sites of the DNA polymerase and the RNase H. HIV-1 RT regions that interact with the template-primer in the binary complex are the 'primer grip' (residues 227–235 of p66), the 'helix clamp' (residues 255–268 and 278–286 of p66), the 'RNase H primer grip' (residues 358–361, 473–476, 501 and 505 of p66, and 395–396 of p51) and the 'template grip' (residues 73–77, 78–83, 86–90 and 141–174 of p66) (Ding et al., 1998). The primer grip is important to maintain the 3′-OH of the primer in an appropriate orientation for the nucleophilic attack on the incoming dNTP.

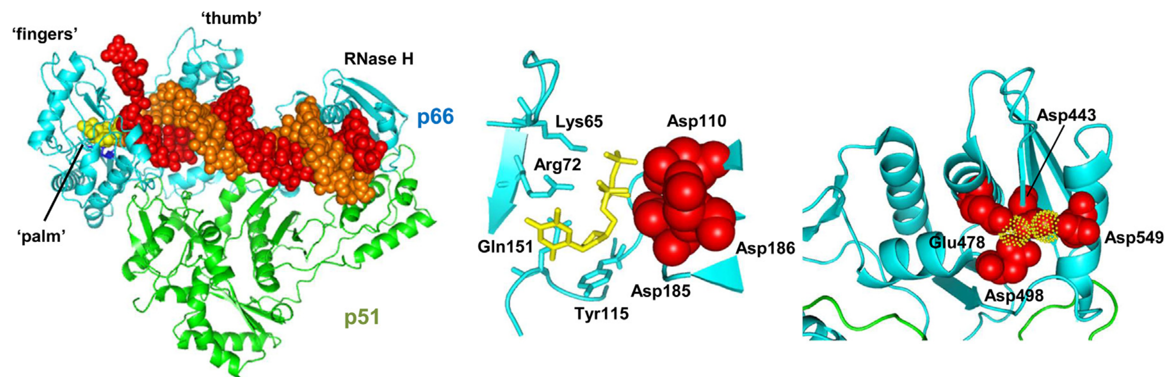

Fig. 5. HIV-1 RT structure and its DNA polymerase and RNase H catalytic sites. (Left) Crystallographic structure of HIV-1 RT bound to dsDNA and an incoming dNTP (PDB file 1RTD). Ribbon representation of the p66 subunit in blue and the p51 subunit in green. The template (red), the primer (orange) and the incoming dNTP (yellow) are represented with spheres. (Middle) Nucleotide binding site in the DNA polymerase domain showing the location of catalytic residues Asp^{110}, Asp^{185} and Asp^{186} (red spheres) and the side-chains of Lys^{65}, Arg^{72}, Tyr^{115} and Gln^{151} (blue sticks). The incoming nucleotide is represented with yellow sticks. (Right) RNase H active site showing the location of Asp^{443}, Glu^{478}, Asp^{498} and Asp^{549} (red spheres), and the coordinating metal ions (dot yellow surfaces). Coordinates were taken from PDB file 3LP1.

The catalytic residues of the DNA polymerase active site are close to the 3′ end of the primer. Asp^{185} and Asp^{186} are part of the conserved YMDD motif and allow proper coordination with metal cofactors and trigger the nucleophilic attack of the 3′-OH of the primer terminus on the α phosphorous of the incoming dNTP (Huang et al., 1998; Mendieta et al., 2008). The incoming dNTP is tightly coordinated by the side-chains of Lys^{65} and Arg^{72} in the fingers subdomain of p66, the main chain amido groups of Asp^{113} and Ala^{114}, and two magnesium cations. Other important residues that delineate the dNTP binding pocket are Tyr^{115}, Phe^{116} and Gln^{151} (Fig. 5). Site-directed mutagenesis studies have shown that Lys^{65} has a major influence on the fidelity of HIV-1 RTs, and its substitution by Arg renders enzymes with more than 8-fold increased fidelity of DNA synthesis (Shah et al., 2000; Mansky et al., 2003; Garforth et al., 2010; Barrioluengo et al., 2011). On the other hand, the aromatic ring of Tyr^{115} acts as a steric gate that allows the incorporation of dNTPs, but prevents the incorporation of rNTPs due to the presence of the 2′-OH in their ribose ring (Gao et al., 1997; Cases-González et al., 2000).

The RNase H activity of HIV RTs degrades the template RNA and the primer tRNA during minus-strand and plus-strand DNA synthesis, respectively, and facilitates the generation of PPTs needed for plus-strand DNA synthesis. The structure of the RNase H domain of HIV-1 RT shows a high similarity to *E. coli* RNase H. It is made of 5 β-sheet structures, flanked by 4 α-helices. Catalytic residues in the active site are coordinated with two manganese cations in the crystal structure, although most likely these are Mg^{2+} *in vivo* (for a detailed description of the catalytic mechanism, see Le Grice, 2012). Crystal structures showed extensive contacts between the HIV-1 RT and nucleic acid next to the RNase H active site (Sarafianos et al., 2001). RT residues in this region are collectively designated 'RNase H primer grip' and interact with the DNA primer 4–9 nucleotides upstream of the scissile bond. The RNase H primer grip determines the trajectory of the RNA strand necessary for its endonucleolytic cleavage.

Apart from HIV RTs, there are crystal structures available for RTs of Moloney MLV (MoMLV) (Das and Georgiadis, 2004; reviewed in Coté and Roth, 2008), XMRV (PDB file 4HKQ; Nowak et al., 2013) and the Ty3 element of *S. cerevisiae* (PDB file 4OL8; Nowak et al., 2014) (Fig. 6). MoMLV and XMRV RTs share 97.5% sequence identity and are monomeric enzymes. Crystal structures of the two full-length proteins bound to dsDNA are available, but in the case of the MoMLV RT the RNase H domain was disordered (Das and Georgiadis, 2004). The structure of XMRV RT bound to dsDNA showed that the nucleic acid interacts with all domains of the protein and sits in a positively-charged substrate-binding channel (Nowak et al., 2013). The protein covers 14 nucleotides of the primer and 16 nucleotides of the template. The architecture of the DNA polymerase active sites of XMRV and HIV-1 RTs are very similar, and the trajectory of the substrate near the catalytic site is superimposable. Differences between both enzymes affect the positioning of the fingers subdomains which are in a more open conformation in binary complexes of HIV-1 RT and nucleic acid. Another remarkable difference relates to the positioning of the RNase H domain, which shows increased mobility in the XMRV RT, in the absence of substrate. However, these differences do not seem to affect strand transfer or PPT generation and removal, since HIV-1 RT can use MoMLV PPT with only slightly affected cleavage specificity (Pullen et al., 1993; reviewed in Champoux and Schultz, 2009).

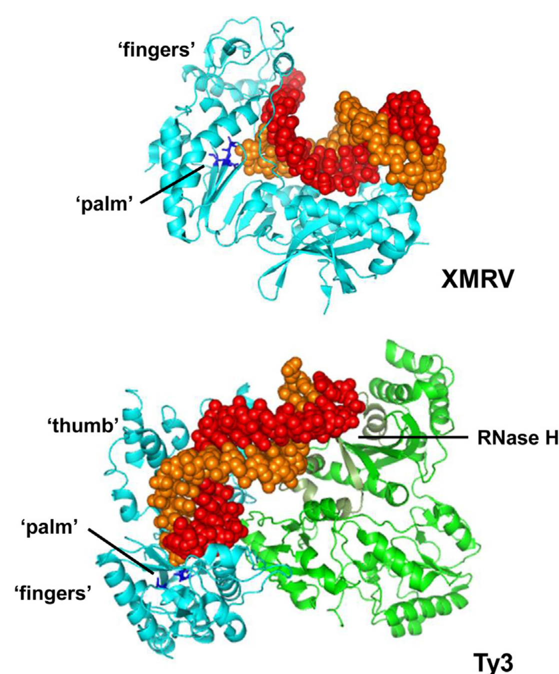

Fig. 6. Crystal structures of the XMRV RT (DNA polymerase domain) and Ty3 RTs complexed with nucleic acid. Ribbons are used to represent the protein backbones (blue for the monomeric XMRV RT and blue and green for the dimeric Ty3 RT). Templates and primers are represented with red and orange spheres, respectively. Coordinates for XMRV and Ty3 RTs were taken from PDB files 4HKQ and 4OL8, respectively.

The Ty3 RT is an asymmetric homodimer of 55-kDa subunits that associates in the presence of the template-primer (Nowak et al., 2014). In the absence of nucleic acid, the Ty3 RT behaves as a monomer (Rausch et al., 2000). The overall conformations of Ty3 and HIV-1 RTs are similar. One of the subunits of the Ty3 RT mimics p51 by adopting a more compact conformation, which also seems to be preferred in the absence of nucleic acid. These findings are also consistent with recently published NMR data showing that HIV-1 RT p66/p66 homodimers are heterodimeric from a structural point of view, and possess conformations very similar to those in mature RT (Sharaf et al., 2014; Zheng et al., 2014; Zheng et al., 2015).

Another notable difference between retroviral RTs and the Ty3 enzyme is that DNA polymerase and RNase H active sites are separated by only 13 base pairs (Lener et al., 2002), although the crystal structure of the Ty3 RT shows that the trajectories of the nucleic acid substrates are very similar to those found in the XMRV RT. Biochemical studies showed that in the Ty3 RT the second carboxylate of the YLDD motif (i.e. Asp^{214}) is not essential for DNA polymerization, although substitutions at this position were found to be lethal for Ty3 transposition (Bibillo et al., 2005). Another unique feature of the Ty3 enzyme is that DNA polymerase and RNase H activities are located in different subunits (Nowak et al., 2014). Still, it is not known whether this is a general property of RTs of LTR retrotransposons.

4.2. Enzymatic properties and characteristics of the DNA polymerase

Retroviral RTs have two different catalytic sites: one for DNA polymerization and a second one for RNA cleavage on RNA/DNA hybrids. Both catalytic sites work simultaneously without being tightly coupled (Li et al., 2016). The DNA polymerization reaction starts with the binding of the RT to the template-primer (usually DNA/DNA or RNA/DNA), followed by the interaction of the incoming dNTP with its binding site in the polymerase active site of the enzyme (Huang et al., 1998). The rate-limiting step in nucleotide incorporation is a conformational change that affects the fingers subdomain of the catalytic subunit. The largest motion within the subdomain occurs at the β3-β4 hairpin loop and facilitates the proper alignment of the α-phosphate of the incoming dNTP with the 3′-end of the primer. The geometry for the nucleophilic attack is facilitated by binding of metal ions with the right size and coordination geometry (Yang et al., 2006). All RTs require two divalent cations for DNA synthesis. Mg^{2+} is the preferred one, although gammaretroviral RTs showed higher specific activity in the presence of Mn^{2+}. Studies carried out with HIV-1 RT showed the different properties of the two Mg^{2+} binding sites (Mendieta et al., 2008). The lower affinity binding site involves interactions with the side-chains of Asp^{110}, Asp^{185} and Asp^{186} and the 3′-OH of the primer. Binding of Mg^{2+} to this site is required to obtain an RT catalytically competent for DNA polymerization.

Kinetic measurements carried out *in vitro* have shown that retroviral RTs are relatively slow in comparison to other DNA polymerases. Although nucleotide incorporation rates depend on the substrate and assay conditions, reported values have been usually in the range of 10–105 nucleotides per second (Kati et al., 1992; Zinnen et al., 1994; Kerr and Anderson 1997; Mendieta et al., 2008; Kisic et al., 2011; Li et al., 2016). Completing minus-strand DNA synthesis in cells may take several hours. In HIV-1-infected 293T cells and in human primary CD4+ T lymphocytes, it has been estimated that this process proceeds at approximately 68–70 nucleotides/min (Thomas et al., 2007). Moreover, compared to other polymerases, RTs show low processivity. In general, RTs may be able to incorporate a few hundred nucleotides in one round of DNA synthesis. However, the presence of RNA secondary structures (e.g. pseudoknots) or specific sequences can cause the dissociation of the enzyme.

Similar to other DNA polymerases that lack 3′ → 5′ exonuclease activity, RTs can add non-templated nucleotides at the 3′ end of nascent DNA *in vitro* (Golinelli and Hughes, 2002a; Golinelli and Hughes, 2002b). The best nucleotide substrate of the reaction was dATP, although the efficiency and base specificity were strongly affected by the sequence at the 3′ end of the blunt-ended duplex. This reaction can take place with RNA/DNA or DNA/DNA complexes. However, the RT has to be in a large excess over the template-primer and requires relatively high dNTP concentrations. Since non-templated nucleotide addition proceeds at a much slower rate than template-dependent polymerization, this reaction may have little impact on retroviral replication *in vivo*. Addition of non-templated nucleotides at the 3′ end of the DNA can facilitate non-specific strand transfer if the 3′ overhangs are complementary to 3′-end sequences of the acceptor strand. It has been shown *in vitro* that RTs of retroviruses and LTR retrotransposons can extend 3′ overhangs of 1, 2 or 3 nucleotides if the reaction takes place in the presence of oligonucleotides showing complementarity at their 3′ end with the overhanging nucleotides (Oz-Gleenberg et al., 2011). This activity (known as ‘clamp activity’) can allow strand transfers onto compatible acceptor strands while synthesizing DNA and can help RTs in bridging over nicks in the copied RNA or DNA templates (Fig. 7).

The RT of the LTR retrotransposon Tf1 has a non-templated nucleotide addition activity that is substantially higher than that of HIV-1 or MLV RTs. The Tf1 RT can add up to seven nucleotides while the other enzymes may incorporate only one dNTP (Kirshenboim et al., 2007; Oz-Gleenberg et al., 2012). This high activity could explain why Tf1 cDNA molecules produced *in vivo* have relatively long extra sequences beyond the highly conserved CA at their 3′-ends (Atwood-Moore et al., 2005).

Another interesting property of retroviral RTs is their capacity to switch templates. Reverse transcription involves at least two strand transfer events facilitated by the presence of repeated sequences in the viral genome. The first strand transfer can be either inter- or intramolecular, while the plus-strand DNA transfer is predominantly intramolecular (reviewed in Basu et al., 2008; see Section 2). A basic feature of the strand transfer mechanism is that RNA degradation by the RT RNase H activity plays an important role by generating the single-stranded DNA region that anneals to the acceptor molecule. The strand transfer process is facilitated by the viral NC protein that enhances minus-strand transfer, by accelerating the annealing of 5′ repeat cDNA to the 3′ repeat sequence of the viral RNA genome.

RTs of retroviruses and retrotransposons (LTR and non-LTR) possess strand displacement activity (Huber et al., 1989; Whiting and Champoux, 1994; Kurzynska-Kokorniak et al., 2007). This activity is needed during plus-strand DNA synthesis and is mediated by the RT-associated helicase activity that facilitates unwinding of the dsDNA during polymerization. Retroviral RTs appear to closely couple dsDNA melting with synthesis-driven translocation of the enzyme, although they do not behave as classical helicases and do not require ATP or any other nucleotide to catalyze processive unwinding at a nick. During reverse transcription, the strand displacement activity helps to eliminate discontinuous plus-strand DNA segments and generates the dsDNA needed for successful integration into the host cell genome. Displacement DNA synthesis is less processive and proceeds at a slower rate than non-displacement synthesis (Whiting and Champoux, 1998). Molecular determinants of the strand displacement activity seem to be associated with specific residues of the fingers subdomain. Thus, mutagenesis studies showed that the deleterious effect on viral replication of amino acid substitutions affecting Phe^{61} in HIV-1 RT and Tyr^{64} in MLV RT can be attributed to a defect in

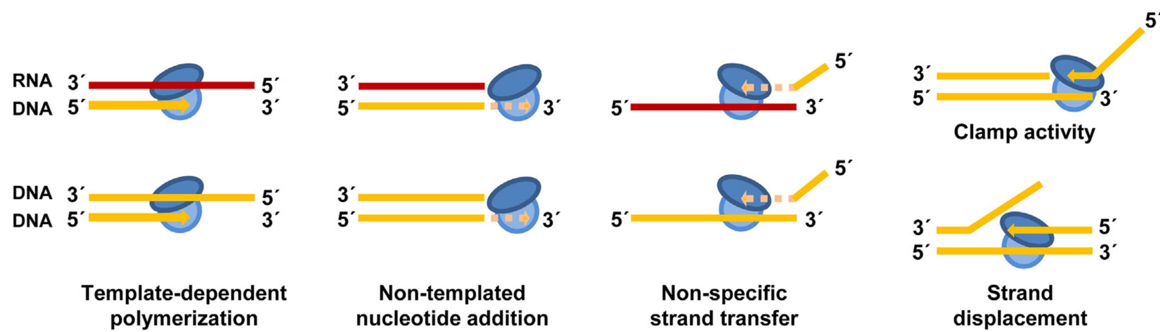

Fig. 7. Substrates used by retroviral RTs during DNA polymerization. RNA is shown in red and DNA in yellow. RT subunits are shown as dark and light blue ovals. Discontinuous DNA strands are used to indicate nucleotide addition on blunt template-primers that result in non-specific strand transfer. For the sake of clarity, clamp and strand displacement activities are shown only for DNA:DNA complexes.

strand displacement activity (Fisher et al., 2003; Paulson et al., 2007).

4.3. RNase H activity

The RNase H activity present in RTs hydrolyzes phosphodiester bonds to produce a 3′-OH and a 5′-phosphate end. This characteristic is important during reverse transcription because it facilitates the generation of RNA fragments that can be used as primers for DNA synthesis (e.g. PPTs). The specificity of this cleavage is different from that shown by pancreatic RNase A and other related endonucleases that leave a phosphorylated 3′ end. As described for the DNA polymerase active site, RNase H activity involves a two-Mg^{2+}-ion catalytic mechanism. RNA/DNA substrates can adopt polymerase-dependent or −independent modes of interaction with the RT (reviewed in Beilhartz and Götte, 2010). Polymerase-dependent (also known as DNA 3′-directed cleavage) occurs when the 3′ end of the DNA strand locates at the polymerase active site, resulting in the cleavage of the RNA 15–20 nucleotides away from the 3′ end of the growing strand (Fig. 8). In this conformation and in the presence of dNTPs, the RT will advance, while its RNase H activity will eventually cut the RNA template. However, it has been estimated that the rate of RNase H cleavage is approximately 5–10 times smaller than nucleotide incorporation (DeStefano et al., 1991; Kati et al., 1992; Li et al., 2016). Other modes of action that involve binding of the RT in an internal region of the DNA are considered polymerase-independent, although many authors make the distinction between RNA 5′-directed cleavage (when the 5′ end of the RNA locates at the polymerase active site) or an internal cleavage, when the nucleic acid ends do not influence RT positioning.

The main function of the RNase H activity in reverse transcription is the elimination of the viral genomic RNA to facilitate the synthesis of viral dsDNA. In principle, specificity is not very important and cleavage preferences are not clearly defined. Therefore, differences between retroviral RTs are relatively small (Nair et al., 2012). However, RNase H specificity is important for the formation of PPT primers and for the elimination of RNA primers used in reverse transcription (i.e. PPT and tRNA). Removal of PPT requires the previous addition of two or three nucleotides at its 3′ end. Then, cleavage occurs at the junction between the RNA primer and the incorporated deoxynucleotides. With some RTs and RNA primers, cleavage specificity is slightly different and can be modulated by the flanking DNA sequences. For example, the tRNA removal reaction catalyzed by the HIV-1 RT leaves a single ribonucleotide (riboA) at the 5′ end of the minus-strand DNA, while in HIV-2, the tRNA is removed completely (Champoux and Schultz, 2009). In addition, it has been shown that some RT inhibitors, such as efavirenz or rilpivirine promote RNase H-mediated trimming of the PPT primer and prematurely block initiation of plus-strand DNA synthesis (Betancor et al., 2015).

Interestingly, the RNase H domain alone is not able to cleave RNA/DNA complexes and requires other elements in the RT structure that are responsible for the proper binding of the template-primer. In agreement with these observations, mutations in the connection subdomain, away from the RNase H active site can have a measurable impact on RNase H activity (Nikolenko et al., 2005; Delviks-Frankenberry et al., 2008; Menéndez-Arias et al., 2011; Betancor et al., 2015).

4.4. Fidelity of DNA synthesis

Retroviral RTs are devoid of $3' \rightarrow 5'$ exonucleolytic proofreading activity and show intrinsic error rates in the range 10^{-4}–10^{-5}, much higher than those reported for eukaryotic replicative DNA polymerases (Matsuda et al., 2000; Kunkel, 2004; McCulloch and Kunkel, 2008; and references therein). Although it has been argued that retroviral genetic variability is a consequence of the high error rate of the RT, there is also an important contribution of the eukaryotic RNA polymerase II as the enzyme responsible for the synthesis of the viral genome using the integrated provirus as template. Transcription errors are very difficult to quantify and current estimates of transcription fidelity are derived from artificial constructs applied to just a few organisms. Despite large differences in error rates reported by several groups, a rough overall average value of 10^{-5} per nucleotide has been suggested (Ninio, 1991), and recent estimates are in agreement with this proposal (Gout et al., 2013; Magnuson et al., 2016; Traverse and Ochman, 2016).

The relative contribution of the viral RT and the host cell RNA polymerase to the high HIV-1 mutation rate has been evaluated in a single cycle of virus replication. These studies support the notion of a larger contribution of the RT, although differences were relatively small, with transcription errors contributing about one third of the error rate found in the progeny virus (O'Neil et al., 2002). On the other hand, viral and host factors can influence mutation rates by different mechanisms. For example, an error rate of 3.4×10^{-5} per nucleotide and replication cycle has been estimated for HIV-1, but the deletion of the *vpr* gene resulted in a 4-fold increase in mutant frequency (Mansky, 1996). Vpr facilitates the incorporation of the nuclear form of uracil DNA glycosylase (UNG2) into HIV-1 virions. UNG2 is a component of cellular DNA repair mechanisms. Interestingly, DNA repair by the nonhomologous end-joining pathway has been associated with mutational escape of HIV-1 from clustered regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9) inhibition in transfected T cells (Wang et al., 2016). Cytidine deaminases such as those of the APOBEC family can be incorporated into HIV and induce deamination of cytidines in the negative strand of the viral DNA, and as a consequence promote $G \rightarrow A$ hypermutation on the plus-strand DNA. APOBEC3-driven mutagenesis occurs mostly at 5′-GG-3′ and

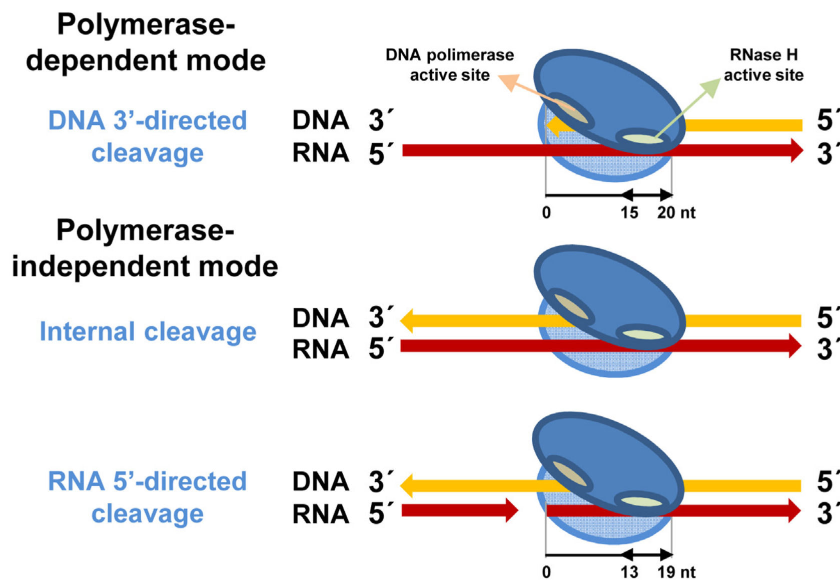

Fig. 8. Retroviral RT binding modes to RNA/DNA hybrids and effects on RNase H cleavage. Polymerase- dependent or -independent binding produce different cleavage patterns and result from different orientations of the RT relative to the template-primer.

5′-GA-3′ dinucleotide contexts (Wood et al., 2009) and contributes to adaptation and evolution in HIV-1 infection (Kim et al., 2014). Other cellular proteins (e.g. adenosine deaminases), viral proteins (e.g. dUTPases) and the available nucleotide levels during reverse transcription may also contribute to the genetic variability found in retroviruses (for further discussion, see Menéndez-Arias, 2013b; Hughes, 2015).

The intrinsic fidelity of purified retroviral RTs has been estimated by measuring mutant frequencies in a reporter gene (e.g. *lacZα*) or in enzymological assays (also known as gel-based assays) (for recent reviews, see Menéndez-Arias, 2009; Menéndez-Arias, 2013b). Reported error rates for HIV-1 RT using the M13mp2 *lacZα* gene as a mutational target range from 6.0×10^{-5} to 6.7×10^{-4} (Roberts et al., 1988; Eckert and Kunkel, 1993; Rezende et al., 1998; Weiss et al., 2004; Matamoros et al., 2008; Álvarez et al., 2013). The large differences observed could be attributed to the diverse conditions used in those assays and the specific RT variant used in the assays. Reported values are underestimates of the mutation rates because assays fail to detect silent mutations or errors that have no effect on the *lacZα* phenotype. Comparative studies have shown that oncoretroviral RTs are about 10–30 times more faithful than lentiviral RTs (Table 1).

Nucleotide incorporation assays carried out with heteropolymeric template-primers have demonstrated that RTs have a good discrimination capacity between correct and incorrect nucleotides. In general, catalytic efficiencies of nucleotide incorporation are 10^3–10^6 times higher for the correct dNTP than for the incorrect one. However, similar assays carried out with a correct nucleotide but using template-primer substrates with either matched or mismatched termini showed that extension efficiencies were 100–10,000 times lower when the template-primer had a 3′ mispaired end. Extension of mismatched 3′ termini has been recognized as a major determinant of the low accuracy of HIV-1 and other retroviral RTs (Perrino et al., 1989; Yu and Goodman, 1992). However, most of those enzymological studies have been carried out with DNA/DNA hybrids. Similar assays carried out with RNA/DNA complexes revealed only some relevant differences in pre-steady-state kinetic assays carried out with specific template-primers. Thus, authors have reported the higher accuracy of HIV-1 RT in the incorporation of C, A, or G opposite A in assays carried out with RNA/DNA duplexes in comparison to DNA/DNA duplexes (Kerr and Anderson, 1997). However, this behavior was not observed with all template-primers, and for some mispairs, differences in accuracy between RNA- and DNA-dependent polymerization were not significant.

As an important target of antiretroviral intervention, the fidelity of HIV-1 RT and the effects of amino acid substitutions in its accuracy have been extensively analyzed. These studies have shown that the major determinants of fidelity locate in the DNA polymerase domain, including: (i) dNTP binding residues (e.g. Lys^{65}, Tyr^{115} or Met^{184}), (ii) residues that interact with the template strand (e.g. Leu^{74} or Val^{75}) or the primer strand (e.g. Met^{230}), and (iii) minor groove binding track residues (e.g. Gly^{262} or Trp^{266}) (for extensive reviews, see Menéndez-Arias, 2002; Menéndez-Arias, 2009). Interestingly, amino acid substitutions conferring resistance to RT inhibitors such as K65R or M184V are known to increase the intrinsic fidelity of the HIV-1 RT (Wainberg et al., 1996; Shah et al., 2000; Barrioluengo et al., 2011). However, increased accuracy is not necessarily related to drug resistance, as observed for other amino acid changes emerging during treatment with RT inhibitors (e.g. L74V, Q151M) (for a review, see Menéndez-Arias, 2009).

Apart from those described above, there are additional determinants of fidelity in the RNase H primer grip of the HIV-1 RT. In this enzyme, Tyr^{501} is part of a conserved motif found in all retroviral RNase H domains except in the RSV RT. The equivalent residue in MLV RT is Tyr^{586} and its substitution by Phe produces a five-fold increase of the mutation rate *in vivo* that is a consequence of the large increase in the frequency of base substitution mutations within homopolymeric adenine and thymidine tracts (Zhang et al., 2002). On the other hand and unexpectedly, HIV-1 RT RNase H inactivation by mutations such as D443N or E478Q has a significant impact on fidelity of DNA-dependent DNA synthesis. Those substitutions produced 2.0- to 6.6-fold increases in fidelity, mainly due to a significant reduction in the base substitution error rates (Álvarez et al., 2013). Although the physiological significance of these findings remains unclear, it was also noted that HIV-1 group O RTs bearing RNase H-inactivating mutations showed higher frameshift error rates, probably as a result of their higher tendency to dissociate from the template-primers.

Table 1
Error rates of recombinant retroviral RTs determined with the forward mutation assay, using M13mp2 *lacZα* as reporter gene.

Retrovirus type	RT	Error rate	Accuracy [a] (fold-change)	HIV-1 RT	References
Alpharetroviruses	AMV	5.9×10^{-5}	9.5	(p66/p51)[b]	Roberts et al. (1988,1989)
Gammaretroviruses	MLV	3.3×10^{-5}	14.3	(p66/p51)[b]	Roberts et al. (1988,1989)
		1.2×10^{-5}	11.7	BH10 (p66/p51)	Barrioluengo et al. (2012)
	XMRV	1.2×10^{-5}	11.7	BH10 (p66/p51)	Barrioluengo et al. (2012)
	FeLV	5.8×10^{-6}	29	BH10 (p66/p51)[c]	Operario et al. (2005)
Lentiviruses	HIV-1 group O	5.5×10^{-5}	2.5	BH10 (p66/p51)	Álvarez et al. (2009)
	SIV_{agm}	2.9×10^{-5}	1.8	HXB2 (p66/p51)	Stuke et al. (1997)
	SIV_{mne}	1.2×10^{-4} (CL8)[d]	1.3	BH10 (p66/p51)[c]	Diamond et al. (2003)
		1.6×10^{-5} (170)	9.7		
	FIV	6.2×10^{-5}	2.8	BH10 (p66/p51)[c]	Operario et al. (2005)
Spumaviruses	PFV	1.7×10^{-4}	0.4	BH10 (p66/p51)	Boyer et al. (2007)

Abbreviations are: HIV-1, human immunodeficiency virus type 1; AMV, avian myeloblastosis virus; MLV, murine leukemia virus; XMRV, xenotropic murine leukemia virus-related virus; FeLV, feline leukemia virus; SIV, simian immunodeficiency virus (agm, African green monkey; mne, pig-tailed macaque); FIV, feline immunodeficiency virus; PFV, prototype primate foamy virus.

[a] Increase relative to the wild-type HIV-1 RT (prototypic HIV-1 group M subtype B strains).

[b] Purified from HIV-1 subtype B virions (unreported strain) (Roberts et al., 1988).

[c] Data for HIV-1 RT were not provided in the referenced papers. For comparative purposes, mutation rates for HIV-1 RT obtained in the same lab were taken from Weiss et al. (2004).

[d] CL8 and 170 are two variants of SIVmne that infected the same macaque. The strain CL8 was obtained earlier in the asymptomatic phase, while 170 was isolated in the late symptomatic phase of the infection.

4.5. Recombination

Retroviruses show high recombination rates. Their virions contain two copies of full-length viral RNA, and the RT can use parts of each one as templates during reverse transcription, thereby facilitating the generation of recombinant variants (Hu and Temin, 1990b). Among retroviruses, recombination rates are higher for primate lentiviruses (e.g. HIV-1, HIV-2 and SIV_{agm}) than for MLV or spleen necrosis virus (Onafuwa et al., 2003; Rhodes et al., 2003; Rhodes et al., 2005; Chen et al., 2006). In HIV-1, recombination is a powerful force that influences the evolution of its genome (for recent reviews, see Smyth et al., 2012; Lau and Wong, 2013). It has been estimated that the global prevalence of recombinant forms is close to 50% of the total infections worldwide (Hemelaar et al., 2011). The generation of recombinants requires the coinfection of the cell by more than one parental virus. Only cells containing different proviruses can generate heterozygous virions. A sequence in the 5′ untranslated region of the RNA acts as a dimerization initiation site (DIS) and facilitates the packaging of two RNAs in the same particle (Moore et al., 2007). This is a palindromic sequence that in HIV-1 is GCGCGC or GUGCAC.

Sequence similarity facilitates template switching and is required for efficient recombination (An and Telesnitsky, 2002; Baird et al., 2006). Recombinant breakpoints are not randomly distributed across the retroviral genome. Hot spots in the genomes of HIV-1 intersubtype recombinants appear to be consistently found at specific regions of the *gag* gene, but also flanking *env* (Fan et al., 2007; Archer et al., 2008; Simon-Loriere et al., 2009; Smyth et al., 2014). However, specific features of those sequences have not been correlated with hot spot propensities. It is assumed that template switching could be facilitated by the presence of nicks in the genomic RNA, or by strong pausing sites during reverse transcription due to the presence of RNA secondary structures (reviewed in Simon-Loriere et al., 2011). These stops enhance the efficiency of template switching because they facilitate the degradation of the RNA by the RNase H activity of the RT and increase the time of residency of the RT in the specific sequence. These ideas constitute the basis of the so-called dynamic copy-choice model which proposes that template switching is influenced by a balance between the polymerase and RNase H activities of the RT (for recent reviews, see Onafuwa-Nuga and Telesnitsky, 2009; Delviks-Frankenberry et al., 2011).

A recombination rate of 1.35×10^{-3} per nucleotide and replication cycle has been estimated using the HIV-1 *gag* gene as a reference (Schlub et al., 2010). This value is two orders of magnitude higher than the mutation rates obtained *ex vivo* (Mansky, 1996). A detailed high-throughput sequencing analysis of HIV collected after a single round of infection in primary T lymphocytes revealed an even higher mutation rate at recombined regions (Schlub et al., 2014). Moreover, recombination-associated mutations were found to represent 15–20% of all mutations occurring during reverse transcription. If mutation induces recombination or vice versa is still an unsolved question that warrants further studies in this area.

4.6. RT inhibition

Thirty years ago, the discovery of AZT (zidovudine or 3′-azido-3′-deoxythymidine) as an effective therapeutic agent against HIV infection represented a major breakthrough in the fight against AIDS. AZT-triphosphate blocks DNA polymerization and its molecular target is the viral RT. Still today HIV-1 RT inhibitors constitute the backbone of widely used anti-AIDS therapies and their development has been a successful and very active field of research for many years (Arts and Hazuda, 2012; De Clercq, 2013). There are two major classes of drugs that block reverse transcription: nucleoside analogues (NRTIs) and nonnucleoside RT inhibitors (NNRTIs). Chemical structures of NRTIs currently approved or in advanced preclinical development are given in Fig. 9. These are compounds with a structure similar to natural nucleosides. They are administered as prodrugs that need to be phosphorylated to their triphosphate forms to act as competitive inhibitors (or alternative substrates) of the RT. Once incorporated into the growing DNA chain, DNA synthesis stops due to the lack of a 3′-OH in their ribose ring. In this class, zidovudine, lamivudine, abacavir, emtricitabine and tenofovir are the most commonly used drugs in the treatment against HIV infection. Tenofovir is an adenosine-monophosphate analogue that is usually combined with emtricitabine and an NNRTI as first-line highly active antiretroviral therapy (HAART).

NRTIs interact with the dNTP binding site of the RT. HIV-1 resistance to these drugs is usually associated with the emergence of amino acid substitutions that increase the catalytic efficiency of incorporation of natural dNTP substrates relative to triphosphate derivatives of the nucleoside analogues. Examples of mutations acting through this discrimination mechanism are M184V or M184I that confer resistance to lamivudine and emtricitabine (Sarafianos et al., 1999), or K65R that confers resistance to tenofovir (Das et al., 2009). This mechanism is also relevant for multidrug resistance as demonstrated for the combination A62V/V75I/F77L/F116Y/Q151M

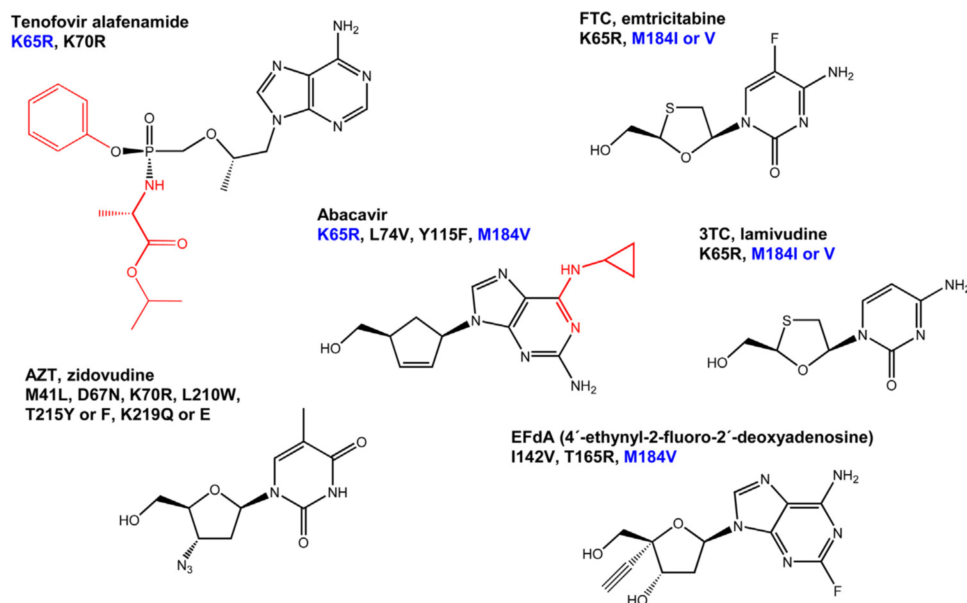

Fig. 9. Chemical structures of nucleoside analogues active against HIV-1. Tenofovir alafenamide, emtricitabine, abacavir, lamivudine and zidovudine are approved drugs commonly used to treat HIV-infected patients. Tenofovir alafenamide is a prodrug currently preferred over tenofovir disoproxil fumarate due to its reduced renal and bone toxicity (Ray et al., 2016). EFdA is a nucleoside analogue in preclinical development. Atoms modified during the conversion of the analogues to metabolically relevant derivatives are indicated in red. Drug resistance associated mutations for each drug are given below. The mutations with the largest impact on resistance are shown in blue. Combinations of TAMs associated with zidovudine resistance are also responsible for cross-resistance with abacavir and tenofovir.

(Deval et al., 2002; Deval et al., 2005). This combination confers resistance to all approved nucleoside analogues except tenofovir (reviewed in Menéndez-Arias, 2008). An alternative mechanism of resistance to NRTIs involves the acquisition of mutations such as M41L, D67N, K70R, L210W, T215F or Y, and K219E or Q, widely known as 'thymidine analogue resistance mutations' (TAMs). HIV-1 RTs carrying different combinations of those mutations are able to excise 3′-terminal chain-terminators from blocked DNA primers in the presence of a pyrophosphate donor (most likely ATP, under physiological conditions) (Meyer et al., 1999). Zidovudine, stavudine and tenofovir are good substrates of the excision reaction, while cytidine analogues (i.e. lamivudine or emtricitabine) are removed very inefficiently (Mas et al., 2002). Substitutions involved in this mutational pathway affect residues in the vicinity of the γ-phosphate of the incoming dNTP (Asp^{67}, Lys^{70} and Lys^{219}), and amino acids located away from the dNTP binding site (Met^{41}, Leu^{210}, Thr^{215}) (Huang et al., 1998). Structural analyses of RT/dsDNA/AZTppppA complexes (containing mutant or wild-type RTs) indicated that the side-chain of Tyr^{215} stabilizes the interaction of the RT with the adenine moiety of ATP, while the side-chains at positions 67, 70 and 219 impose constraints to the β3-β4 hairpin loop and facilitate the proper alignment of the pyrophosphate moiety in the excision reaction (Tu et al., 2010). More information and a detailed analysis of the molecular mechanisms of resistance to NRTIs can be found in previously published reviews (Menéndez-Arias, 2008; Das and Arnold, 2013a,b; Menéndez-Arias, 2013a).

An interesting NRTI in advanced preclinical development is the 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) (Fig. 9). This drug blocks HIV replication in peripheral blood mononuclear cells (PBMCs) at picomolar concentrations, and therefore it is orders of magnitude more potent than any other approved NRTI (Ohrui, 2006). EFdA is also effective against wild-type and clinical drug-resistant HIV strains (Kawamoto et al., 2008). The drug can act as a *bona fide* chain terminator, but it can also allow the subsequent incorporation of one nucleotide that in this case would produce a mismatch that would be difficult to extend. This mechanism of inhibition is known as delayed chain termination and prevents excision of the inhibitor by the ATP-dependent phosphorolytic pathway (Michailidis et al., 2014). Crystallographic analysis of HIV-1 RT complexes containing the inhibitor reveal that the high potency of the NRTI can be attributed to additional interactions established between the 4′-ethynyl group and a hydrophobic pocket in the RT polymerase active site, not observed with other nucleotide analogues or natural dNTPs (Salie et al., 2016).

NNRTIs are small hydrophobic molecules that act as allosteric inhibitors of HIV-1 RT (Fig. 10). They bind to a pocket located about 10 Å away from the dNTP binding site. Only HIV-1 isolates of the major phylogenetic group (including the most abundant subtypes B and C) are inhibited by approved NNRTIs such as nevirapine, efavirenz, etravirine or rilpivirine. Almost all retroviral RTs (including those of HIV-1 group O, HIV-2 and SIV) lack the NNRTI binding pocket and are resistant to these inhibitors. High-level resistance to these inhibitors can be developed easily, particularly to nevirapine and efavirenz which are considered as first-generation inhibitors. Ten or more single amino acid substitutions are known to confer by themselves >40-fold increased resistance to nevirapine (e.g. K103N, Y181C, G190A) or efavirenz (e.g. K101P, K103N, Y188L) in phenotypic assays (reviewed in Menéndez-Arias et al., 2011; Menéndez-Arias, 2013a).

Next-generation inhibitors such as etravirine and rilpivirine show a higher genetic barrier and studies *in vitro* revealed that at least two amino acid substitutions were needed to achieve high-level resistance to those drugs. Examples are V179F/Y181C, V179F/Y181I and Y181I/M230L in the case of etravirine (Azijn et al., 2010; Javanbakht et al., 2010). However, clinical studies have shown the emergence of E138K and M184I in patients failing treatment with rilpivirine (combined with the NRTIs tenofovir and emtricitabine) (Cohen et al., 2011; Molina et al., 2011). E138K and other amino acid changes at this position appear to be major determinants of resistance for both etravirine and rilpivirine (Asahchop et al., 2013). A similar diarylpyrimidine known as dapivirine is in an advanced stage of development as a microbicide for women (Baeten et al., 2016). K103N, Y181C and G190A are frequently found in transmitted HIV-1, particularly in resource-limited countries (Rhee et al., 2015; Avila-Rios et al., 2016). Doravirine, a novel NNRTI in clinical trials, has a relatively high genetic barrier *in vitro*

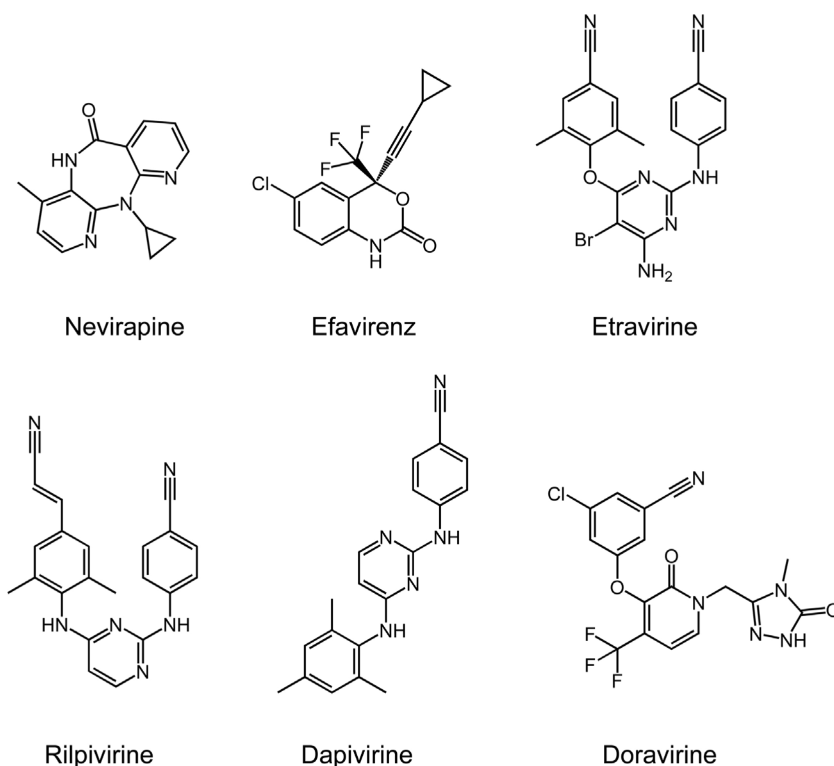

Fig. 10. Chemical structures of nonnucleoside RT inhibitors.

and shows remarkable inhibitory activity against HIV-1 strains bearing the most prevalent NNRTI resistance-associated mutations (Feng et al., 2015). In addition, recent work reveals that mutations that reduce the potency of doravirine and rilpivirine are non-overlapping (Smith et al., 2016), thereby increasing the interest in this drug.

Inhibitors of the RNase H activity have not yet been developed into effective antiretroviral drugs, despite their expected potency against drug-resistant strains. The structural similarity between the active sites of HIV-1 IN and RNase H and the development of raltegravir as an IN inhibitor stimulated new efforts in this area of research that led to the discovery of many RNase H inhibitors active in *in vitro* assays. Newly identified compounds can be classified as active site or allosteric RNase H inhibitors (for a review, see Ilina et al., 2012). Active site inhibitors are based on pharmacophores that interact with the two cations of the RNase H active site. Compounds having micromolar or submicromolar potency against the HIV-1 RNase H activity are based on pharmacophore scaffolds such as diketo acids, N-hydroxy naphthypyridinones, N-hydroxyimides, pyrimidinol carboxylic acids, and tropolones (Kirschberg et al., 2009). GSK5724 (a pyrimidinol carboxylic acid) was identified as a very potent RNase H inhibitor *in vitro* (IC_{50} = 3 nM) and had strong antiviral activity in cell culture (IC_{50} = 10 nM) (Johns and Velthuisen, 2011). The drug also showed sub-micromolar inhibitory activity against the viral IN. Many RNase H active site inhibitors, including the well-known β-thujaplicinol (a tropolone derivative) show slow binding kinetics while dissociating rapidly. GSK5750 is a novel 1-hydroxy-pyridopyrimidinone analogue that forms long lasting complexes with HIV-1 RT, and binds the enzyme with an equilibrium dissociation constant (K_d) of ~400 nM (Beilhartz et al., 2014).

A few compounds have been identified as allosteric inhibitors of the RT RNase H. These drugs do not bind directly to the active site and cannot be displaced by nucleic acid substrates. Thiocarbamates, 1,2,4-triazoles, acylhydrazones and vinylogous ureas are pharmacophores found in the most effective inhibitors of this class. The vinylogous urea NSC727447 appears to interact with the thumb subdomain of p51 at the interface with the RNase H domain of HIV-1 RT (Chung et al., 2012). Interestingly, acylhydrazone derivatives such as the dihydroxy benzoyl naphthyl hydrazone (DHBNH) and other related compounds were shown to inhibit both HIV-1 RNase H and DNA polymerase activities (Borkow et al., 1997; Gong et al., 2011; Corona et al., 2016). The specific mechanisms involved in this dual inhibition are not clear and could be diverse. For example, the isatin-based compound RMNC6 seems to bind at different sites in the polymerase and RNase H domains of the RT (Corona et al., 2016). In contrast, DHBNH binds to the DNA polymerase domain in a way that alters the trajectory of the RNA/DNA hybrid causing defects in the RNase H activity (Himmel et al., 2006).

5. Reverse transcription in *Hepadnaviridae*

Hepadnaviruses are small hepatotropic viruses that infect mammals and birds. Hepatitis B virus (HBV) is one of the most important pathogens in humans. The HBV polymerase (or P protein) is a multifunctional enzyme of approximately 90 kDa that contains four domains: (i) a terminal protein (TP) domain, (ii) a spacer domain, (iii) an RNA-dependent and DNA-dependent DNA polymerase domain, and (iv) an RNase H domain. The DNA polymerase and RNase H domains are structurally similar to the equivalent ones found in retroviruses, such as HIV-1 or MLV (Fig. 11), with conserved motifs such as YMDD in the catalytic active site of the polymerase or the conserved D-E-D-D motif in the RNase H domain (reviewed in Menéndez-Arias et al., 2014; Hu and Seeger, 2015). The catalytic role of Asp^{702}, Glu^{731} and Asp^{750} in the RNase H domain has been inferred from sequence alignments and molecular modelling with HIV-1 RT, and demonstrated *in vitro* by mutational studies (Tavis et al., 2013). The fourth residue is more difficult to assign based on comparative sequence analysis. Nevertheless, it has

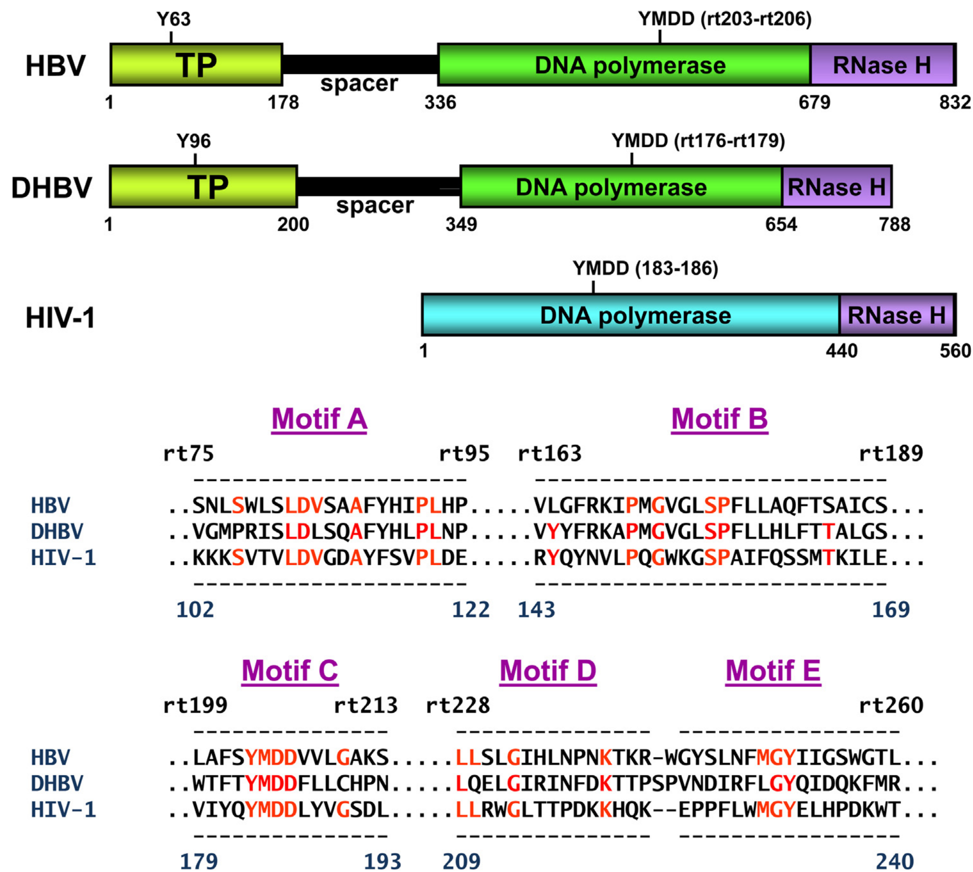

Fig. 11. Hepadnaviral polymerases and HIV-1 RT domain organization and conserved motifs in their DNA polymerase domains. Sequences correspond to HBV genotype A (subtype adw2), duck hepatitis B virus (DHBV) (white Shanghai duck/S31) and HIV-1 group M subtype B isolates BH10 (GenBank accession numbers AM282986, M32991 and M15654, respectively). Alignments are based on those reported by Poch et al. (1989). For the HBV and DHBV polymerases, the amino acid positions indicated after the YMDD motif were assigned according to the notation of Stuyver et al. (2001). This notation has been used in the alignments shown below and corresponds to positions in the DNA polymerase domains of both enzymes.

been shown that mutating Asp^{790} leads to an RNase H-deficient phenotype (Ko et al., 2014).

The TP domain of the viral polymerase is needed for initiation of reverse transcription and is unique to hepadnaviruses. The OH group in a specific Tyr residue in this domain (Tyr^{63} in HBV polymerase) is used as a 3′-hydroxyl end of a nucleic acid primer (reviewed in Nassal, 2008; Clark and Hu, 2015). The template molecule is a pgRNA that contains at its 5′ end an RNA stem-loop structure termed epsilon (ε). The pgRNA has a structure similar to that of cellular mRNAs with a cap at its 5′ end and a polyadenylated tail at its 3′ end. In addition it has repeated sequences, known as direct repeats 1 and 2 (DR1 and DR2) that facilitate strand transfer during reverse transcription. These sequences are located at the 5′ end (DR1) and at the 3′ end of the pgRNA (DR2 and a second DR1) (Fig. 12).

The interaction of the polymerase with ε triggers its coencapsidation with the pgRNA in newly forming nucleocapsids, and the initiation of viral DNA synthesis. The DNA polymerase and RNase H activities are not required for RNA packaging (Hirsch et al., 1990; Jones et al., 2014). Cellular proteins belonging to the family of molecular chaperones (i.e. Hop, p23, Hsc70, Hsp40 and Hsp90) are required for the initial binding of the polymerase to the ε RNA (Hu and Seeger, 1996; Hu et al., 1997; Hu et al., 2004; reviewed in Hu and Seeger, 2015). HBV RNA packaging and protein priming is also dependent on the presence of a 5′ cap structure, which has to be separated by less than 70 nucleotides from the ε structure (Jeong et al., 2000; Jones et al., 2012). Synthesis of the DNA genome of mature hepadnaviruses with the pgRNA template takes place inside viral cores, in the host cell cytoplasm. Structural studies revealed that these cores have an asymmetric structure when filled with RNA, although the polymerase sits on an ordered RNA lattice where the 5′ and 3′ ends of the pgRNA locate close to each other stabilizing a circular conformation of the pgRNA (Abraham and Loeb, 2007; Wang et al., 2014).

The protein priming reaction involves the formation of a covalent phosphotyrosyl bond between the Tyr of the TP domain and dGMP. Two to three additional nucleotides are then added to generate a sequence GTAA, complementary to the UUAC sequence found in the bulge of the ε RNA stem-loop in duck HBV, or GAA complementary to UUC in the case of human HBV. This reaction takes place in the presence of Mg^{2+} and is completely dependent on the viral ε structure in these conditions (Jones et al., 2012). However, an ε-independent protein-primed transferase activity has also been detected *in vitro* in the presence of low concentrations of Mn^{2+}. For this activity, the viral polymerase could use all four dNTPs, although dTTP was the preferred substrate (Jones and Hu, 2013).

The GTAA sequence obtained in the ε-dependent reaction, which is covalently attached to the RT protein, is translocated (minus-strand primer transfer) to anneal with a complementary sequence motif located at the 3′ end of the pgRNA, in the 10–12-nucleotides long DR1 region. This process is facilitated by the presence of additional elements in the RNA (i.e. φ and ω) that stabilize the circular conformation of the pgRNA and promote transfer of the short minus-strand DNA (Abraham and Loeb, 2007). The same mechanism operates for human HBV although in this case protein priming initiates with the sequence GAA (reviewed in Nassal, 2008;

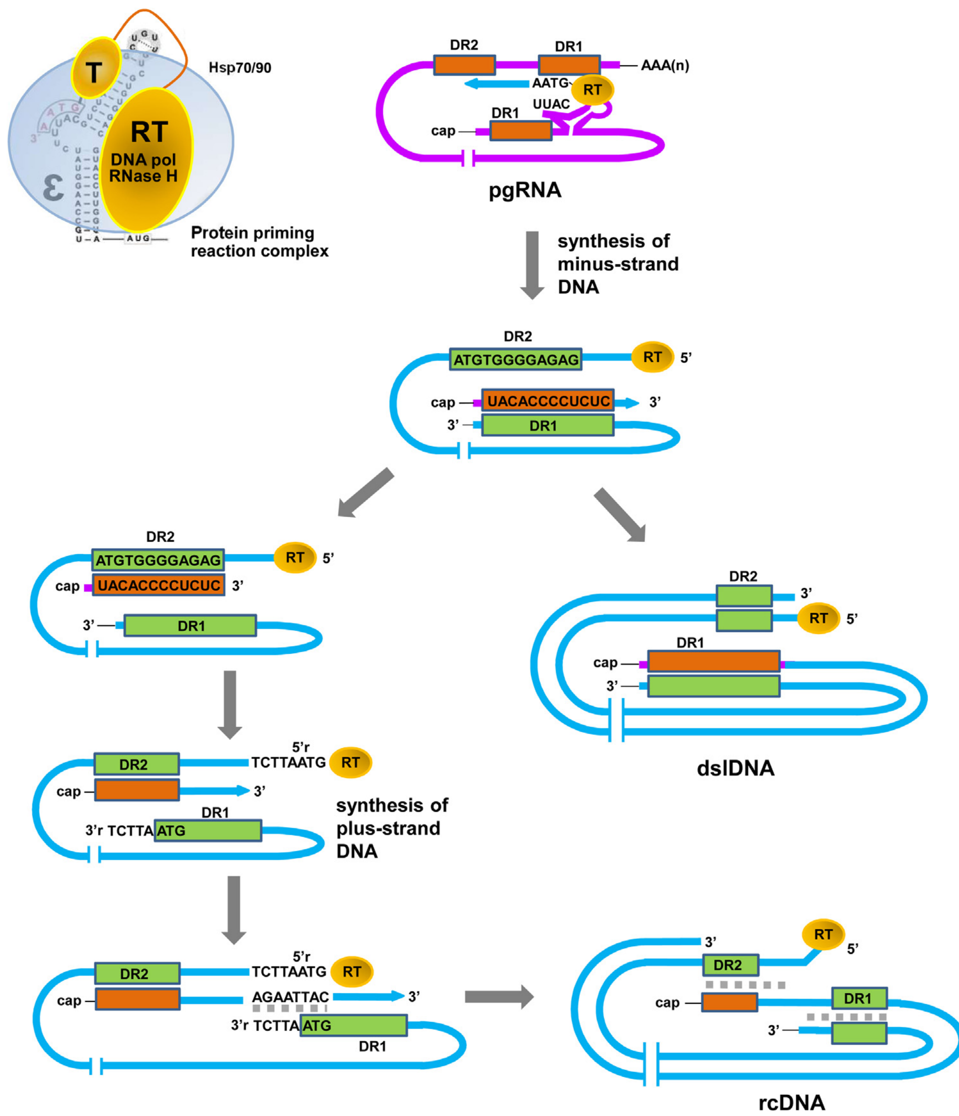

Fig. 12. Reverse transcription in *Hepadnaviridae*. The model shows the formation of rcDNA and dslDNA from pgRNA. A schematic representation of the ribonucleoprotein complex necessary for the protein priming reaction is shown in the upper left panel. Synthesis of minus-strand DNA (depicted in blue) using pgRNA (purple) as template leads to an intermediate that contains a DR1 primer that could be elongated to generate dslDNA. Alternatively, the DR1 primer can anneal to a DR2 sequence at the 5′ of the minus-strand DNA and after elongation and additional strand transfer events generate the rcDNA molecule.

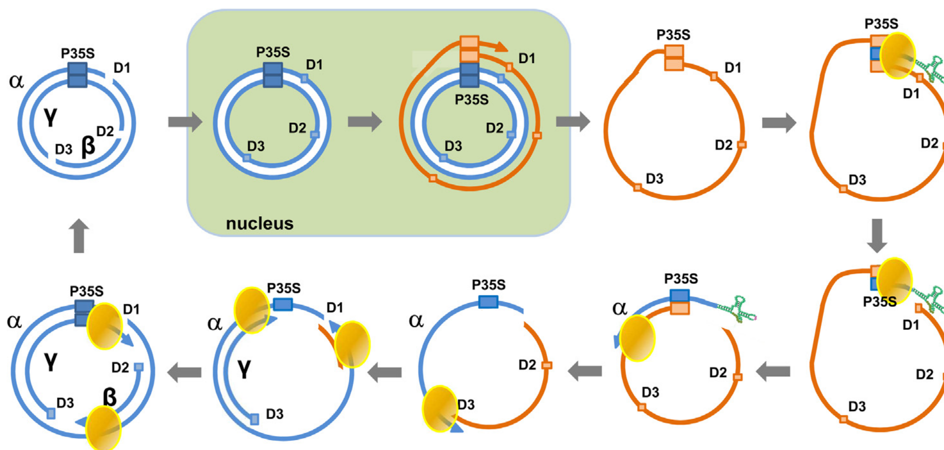

Fig. 13. Reverse transcription in *Caulimoviridae*. Infectious viruses contain viral DNA (shown in blue), consisting of three fragments designated as α, β and γ. Inside the nucleus, a viral RNA (shown in red) is synthesized by the transcription machinery of the cell. Reverse transcription takes place in the cytoplasm where a $tRNA^{Met}$ acts as a primer to initiate the process. D1, D2 and D3 are discontinuities that correspond to sequences rich in purines and used as primers to synthesize the β and γ strands of the DNA. The viral RT is represented by an orange spheroid.

Hu and Seeger, 2015). After template switching, the minus-strand DNA synthesis reaction proceeds until the 5′ end of the pgRNA, with the concomitant degradation of the template by the RNase H activity of the P protein.

Plus-strand DNA synthesis is primed by a capped RNA oligonucleotide derived from the 5′ end of the pgRNA. This 18-nt long oligomer contains part of the DR1 sequence and could either be used as a primer, *in situ*, to initiate the synthesis of the plus-strand DNA, or as occurs in about 80% of the cases, translocate to the DR2 region at the 5′ end of the minus-strand DNA. By using this alternative priming site, the viral polymerase completes plus-strand DNA synthesis all the way up to the 5′ end of the minus-strand DNA, while an additional template switch facilitates the circularization of the genome. Redundant sequences (r) at the 5′ and 3′ ends of the minus-strand DNA facilitate this process. As a result, most infectious virions contain rcDNA, although a minority may have dslDNA (Hu and Seeger, 2015).

One of the hallmarks of hepadnavirus infection is the formation of cccDNA. This episomal DNA serves as template for all viral RNA transcription and is stable in nuclei of infected cells (Nassal, 2015). The mechanism for cccDNA formation has not been clearly established. The cccDNA originates from rcDNA after: (i) full extension of plus-stranded DNA, (ii) removal of the P protein from the 5′ end of the minus-stranded DNA, of the 18-nt long RNA primer used in plus-stranded DNA synthesis, and of one of the 9-nucleotide-long redundant sequences on minus-strand DNA, and (iii) ligation of both DNA strands forming the cccDNA. It is not clear whether the viral polymerase has a role in this conversion of rcDNA to cccDNA. The participation of cellular enzymes involved in DNA repair pathways (e.g. endonucleases, DNA polymerase κ and different ligases) is possible, and a tyrosyl-DNA phosphodiesterase (known as Tdp2) has been recently implicated in the release of the RT from the 5′ end of minus-strand DNA (Jones et al., 2012; Jones and Hu, 2013; Königer et al., 2014; Cui et al., 2015).

5.1. Biochemical properties of the HBV polymerase

Despite being an important target for antiviral therapy and its essential role in virus replication, structural and biochemical studies with the HBV polymerase have been limited by difficulties in obtaining sufficient quantities of the recombinant enzyme. Duck HBV polymerase has been used as a model system although it shares only about 20% sequence identity. Duck HBV polymerase expressed by *in vitro* translation in the rabbit reticulocyte lysate is active in authentic protein priming (Wang and Seeger, 1992). Studies using this *in vitro* system led to the identification of host cell chaperones required for polymerase functions (Hu and Seeger, 1996; Hu et al., 1997). Truncated duck HBV polymerase has been obtained in *E. coli*. In the presence of metal ions and after reconstitution with recombinant host chaperones and an ATP-regenerating system, this enzyme showed *in vitro* priming and elongation activity (Hu and Anselmo, 2000; Beck and Nassal, 2001; Hu et al., 2002; Beck and Nassal, 2003). Unfortunately, human HBV polymerase obtained from rabbit reticulocyte lysates did not show enzymatic activity and the enzyme obtained in insect cells with baculovirus systems had very low protein-priming activity, detectable in the absence of the ε RNA element (Lanford et al., 1995; Lanford et al., 1997). HBV polymerase has recently been expressed in mammalian cells, together with the viral ε RNA. When purified as a protein-RNA complex, it showed authentic protein priming activity *in vitro* (Jones et al., 2012). Interestingly, a protein-primed transferase activity independent of the ε RNA element was also discovered in the human HBV polymerase (Jones and Hu, 2013).

Recently, the TP domain (residues 1–192) and the RT-RNase H domains (residues 303–778) of the human HBV polymerase have been successfully expressed in *E. coli*, purified in high yields and solubilized and refolded in buffers containing an amphipathic carbohydrate polymer (Vörös et al., 2014). These domains were found to have a high α-helical content. This approach may provide new possibilities for structural and biochemical characterization of the viral polymerase. As in the case of retroviral RTs, the HBV polymerases are devoid of proofreading activity and are expected to display relatively low fidelity. Mutation rates for duck HBV replication were estimated to be around 2×10^{-5} per nucleotide and generation (Pult et al., 2001), and as in the case of HIV-1 and other retroviruses, APOBEC proteins could be packed into the viral cores and affect reverse transcription efficiency and its error rate (Baumert et al., 2007; reviewed in Janahi and McGarvey, 2013).

The RNase H domain of human HBV shares 23% identity with the equivalent in HIV-1 RT (Tavis et al., 2013). An extensive biochemical characterization of the HBV RNase H activity has been possible after its successful expression and characterization as a fusion protein with a maltose-binding domain at the N-terminus and a hexahistidine tag at the C-terminal end (Villa et al., 2016). In the presence of Mg^{2+} and ATP, authors obtained monomeric recombinant enzyme with endonucleolytic activity. A minimal size of 14 nucleotides was necessary for cleavage of RNA/DNA hybrids. The HBV RNase H also showed processive 3′ → 5′ exoribonuclease activity but its catalytic rate was reduced in comparison with that observed for the endonucleolytic activity. HBV is genetically diverse and eight or nine genotypes have been identified. Active RNase H has been obtained from genotypes B, C, D and H.

5.2. HBV polymerase inhibition

Approved nucleoside/nucleotide inhibitors of the HBV polymerase include lamivudine, adefovir, telbivudine, entecavir and tenofovir. In addition, clevudine has been licensed in South Korea and the Philippines for treating chronic hepatitis B. Development of those drugs has been facilitated in part by large efforts in antiretroviral therapy, and the functional and structural similarities between the HBV polymerase and the HIV-1 RT (Michailidis et al., 2012). Lamivudine and tenofovir are drugs approved for clinical use against both HIV and HBV infection. In addition, the anti-HIV drug emtricitabine has not been approved for treatment of HBV infection, but shows inhibitory activity against HBV replication (reviewed in Menéndez-Arias, 2013a; Menéndez-Arias et al., 2014).

All these drugs need to be converted into triphosphate derivatives in order to become substrates of the HBV polymerase. In general, those compounds act as competitive inhibitors (reviewed in Clark and Hu, 2015). Thus, entecavir triphosphate is a dGTP analogue that inhibits reverse transcription initiation by interfering with the protein priming reaction. Entecavir triphosphate has a 3′-OH in its ribose ring and is a delayed chain terminator (Jones et al., 2013). After priming, the incorporation of A can be impaired by the incorporation of tenofovir diphosphate or adefovir diphosphate. These drugs act as competitive inhibitors that block DNA synthesis. Cytidine analogues (i.e. lamivudine and emtricitabine) lack a 3′-OH in their ribose ring and block DNA polymerization after translocation of the polymerase complex to the DR1 sequence near the 3′ end of the pgRNA. Interestingly, clevudine triphosphate is not incorporated into the DNA and does not affect polymerase binding to ε RNA. It inhibits protein priming and subsequent polymerization steps in a non-competitive manner, probably by distorting the polymerase active site (Jones et al., 2013). As described for HIV-1 RT, resistance to nucleoside/nucleotide inhibitors of HBV polymerase is usually associated with amino acid substitutions at the vicinity of the DNA polymerase active site. Mutations such as L180M, M204I or V, A181T or V and N236T are commonly associated with resistance to approved nucleos(t)ide inhibitors (for a review, see Menéndez-Arias et al., 2014). NRTIs in development include novel

prodrugs of tenofovir and adefovir, as well as new drugs such as besifovir or 4′-modified nucleosides (e.g. 4′-*C*-cyano-2-amino-2′-deoxyadenosine and 4′-*C*-cyano-2-amino-2′-deoxyguanosine) that were shown to be active against HBV strains resistant to currently approved therapies (Block et al., 2015; Takamatsu et al., 2015).

The RNase H is also a promising target that remains unexploited. A number of inhibitors with IC_{50} values in the low micromolar range have demonstrated efficacy against HBV RNase H (reviewed in Tavis and Lomonosova, 2015). The most effective were napthyridinones ($IC_{50} \sim 4.1$–$5.7\,\mu M$) and α-hydroxylated tropolones such as the β-thujaplicinol (IC_{50}s range from 2.3 to 5.9 μM depending on the HBV genotype), although they also showed some inhibitory activity against the human RNase H (Hu et al., 2013; Tavis et al., 2013; Cai et al., 2014; Lu et al., 2015).

6. Reverse transcription in plant pararetroviruses

Caulimoviridae are plant pararetroviruses that replicate through transcription and reverse transcription. Unlike retroviruses, the viral DNA obtained after reverse transcription does not integrate into the host cell (reviewed in Rothnie et al., 1994; Hohn and Rothnie, 2013). Their replicative cycle has similarities with that of hepadnaviruses, accumulating episomal copies (minichromosomes) in the host cell nucleus. The best known viruses of this family are the cauliflower mosaic virus (CaMV) (Haas et al., 2002) and the rice tungro viruses (Hull, 1996). CaMV capsids contain double-stranded circular DNA of about 8 kb. This DNA has an open circular form because it contains interruptions in both plus and minus DNA strands, again reminiscent of hepadnavirus rcDNA. Inside the nucleus, these discontinuities are repaired by the host cell machinery and the supercoiled episomal molecule, like hepadnavirus cccDNA, is used to synthesize two polyadenylated transcripts, known as 35S and 19S RNAs. The 35S RNA shows redundancy at its 5′ and 3′ ends, with identical copies of its promoter at both ends. This RNA is used for the expression of five viral proteins including the RT (known as P5 in the CaMV). P5 is a 78-kDa protein with aspartyl protease, DNA polymerase and RNase H domains. Unlike in the case of retroviral *pol*-derived polyproteins, it lacks the integrase domain. An autocatalytic reaction releases the protease from P5 (Torruella et al., 1989). Viral DNA and protein synthesis occurs in the cytoplasm in electron dense inclusion bodies.

Reverse transcription starts after annealing of a $tRNA^{Met}$ initiator at a site close to a discontinuity (D1) near the 5′ end of the 35S RNA (Fig. 13). DNA synthesis proceeds while the RNase H activity of P5 removes RNA from the RNA/DNA hybrid, leaving behind the DNA. The newly synthesized DNA binds the 35S RNA promoter at the 3′ end of the template, and after this switch DNA polymerization continues. At the same time, purine-rich regions at discontinuities D2 and D3 are resistant to RNase H activity and are used to synthesize DNAs with plus-strand polarity that because of their discontinuities have an open circular conformation. Reverse transcription in rice tungro viruses is similar, but only two discontinuities were observed in these viruses. The rice tungro virus polymerase has been expressed in insect cells using a baculovirus system. Polypeptides of 62 and 55 kDa were obtained, both with DNA polymerase activity, although only the 55 kDa form had RNase H activity (reviewed in Hull, 1996). *Caulimoviridae* RTs lack significant strand displacement activity and produce only small overhangs at the sites where discontinuities are observed. So far, there is hardly any information on the biochemical characteristics and structure of CaMV P5 or closely related proteins.

7. Conclusions and perspectives

Evidence summarized in this article reveals the complexity of the reverse transcription process in many viruses. Viral RTs are quite remarkable in their ability to exploit their DNA polymerase and RNase H activities. By using different types of substrates and their capacity to switch templates, viral RTs are able to produce unique and sophisticated dsDNAs which facilitate virus persistence in infected cells (e.g. LTR-containing proviruses in retrovirus, or cccDNA in hepadnaviruses). After their discovery in 1970, retroviral RTs received attention due to their expected role in RNA tumor virus pathogenicity. However, the discovery of HIV-1 as the etiological agent causing the acquired immunodeficiency syndrome (AIDS) and the need of drugs to block HIV replication fueled research on reverse transcription. During the last four decades, HIV replication has remained as an important research area and RT inhibitors constitute the backbone of current antiretroviral treatments. Part of this knowledge has been transferred to HBV, and several nucleoside analogues acting on the HBV polymerase have been identified and approved for treatment of HBV infections.

Despite the significant progress there are still important gaps in our understanding of the reverse transcription process. In retroviruses, and particularly in HIV, the contribution of viral and host factors to triggering the events that initiate reverse transcription (e.g. uncoating) is still poorly understood. Little is known of the formation of the reverse transcription complex and how this is transformed into a preintegration complex while the viral dsDNA synthesis is being completed. In the case of hepadnaviruses, progress has been slower and very important issues remain unsolved. For example, three-dimensional structures of HBV polymerases are not available and efforts to improve antiviral drugs are hampered by the lack of high-resolution structural information. In addition, we know only the basics of the reverse transcription process, and fates and relevance of its products need to be further studied. This is even more evident in plant pararetroviruses and LTR retrotransposons where biochemical studies are not fueled by the necessity to combat deadly diseases for humans.

Future studies on reverse transcription will be necessary for a better control of viral infections and may provide additional targets of antiviral intervention. Besides, RTs can be also useful tools for biotechnological applications, nowadays especially relevant with the advent of;1; massive sequencing technologies.

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