CMLS, Cell. Mol. Life Sci. 58 (2001) 1246–1262
1420-682X/01/091246-17 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Reverse transcription of retroviruses and LTR retrotransposons

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Received 15 March 2001; received after revision 12 April 2001; accepted 12 April 2001

Abstract. Retroelements are mobile genetic entities that replicate via reverse transcription of a template RNA. A key component to the life cycle of these elements is the enzyme reverse transcriptase (RT), which copies the single-stranded genomic RNA of the element into a linear double-stranded DNA that is ultimately integrated into the host genome by the element-encoded integrase. RT is a multifunctionnal enzyme which possesses RNA-dependent and DNA-dependent DNA polymerase activities as well as RNase H activity that specifically degrades the RNA strand of RNA-DNA duplexes. At some stages of the replication a strand-displacement activity of RT is also necessary. All activities are essential for the conversion of single-stranded genomic RNA into the double-stranded preintegrative DNA. This review focuses on the role of RT in the different steps of the replication process of retroelements. The features of retrotransposon replication which differ from the retroviral ones will be emphasized. In a second part of the review, the biochemical and enzymatic properties of two newly characterized retrotransposon RTs will be described. The role of the integrase domain in reverse transcriptase activity of some retroviral and retrotransposon RTs will be discussed.

Key words. Reverse transcriptase; retrotransposons; retroviruses; Ty; *Saccharomyces cerevisiae;* IN-RT fusion protein.

Introduction

Retroelements are mobile genetic entities that replicate via reverse transcription of a template RNA. A key component to the life cycle of these elements is the enzyme RT which copies the single-stranded genomic RNA of the element into a linear double-stranded DNA that is ultimately integrated into the host genome by the element-encoded integrase. Sequence comparisons from more than 80 reverse transcriptases has divided the retroelements into two major groups [1–3]. One group contains the eukaryotic retrotransposable elements without long-terminal repeats (non-LTR retrotransposons) and the bacterial and organellar elements (group II introns, Mauriceville plasmid of mitochondria); a second group contains the LTR retrotransposons and the retroviruses. The LTR retrotransposons segregate phylogenetically into the Ty1-*copia* family and the Ty3-*gypsy* family. Structurally, the two families are distinguished by the order of the domain encoded within the *pol* gene: the order is protease-reverse transcriptase-integrase (PR-RT-IN) for the Ty3-*gypsy* group, and it is PR-IN-RT for the Ty1-*copia* group. The LTR retrotransposons share similarities with retroviruses both in their genomic arrangement (fig. 1) and in the mechanism of transposition [4–8]. Like retroviruses, LTR retrotransposons replicate through reverse transcription of their genomic RNA, and they encode proteins with homology to the Gag and Pol proteins of retroviruses. The main difference is that LTR retrotransposons do not encode an envelope gene (env) and therefore are not infectious, i.e they carry on their replication cycle within a single cell. Much of what is known about the mechanism

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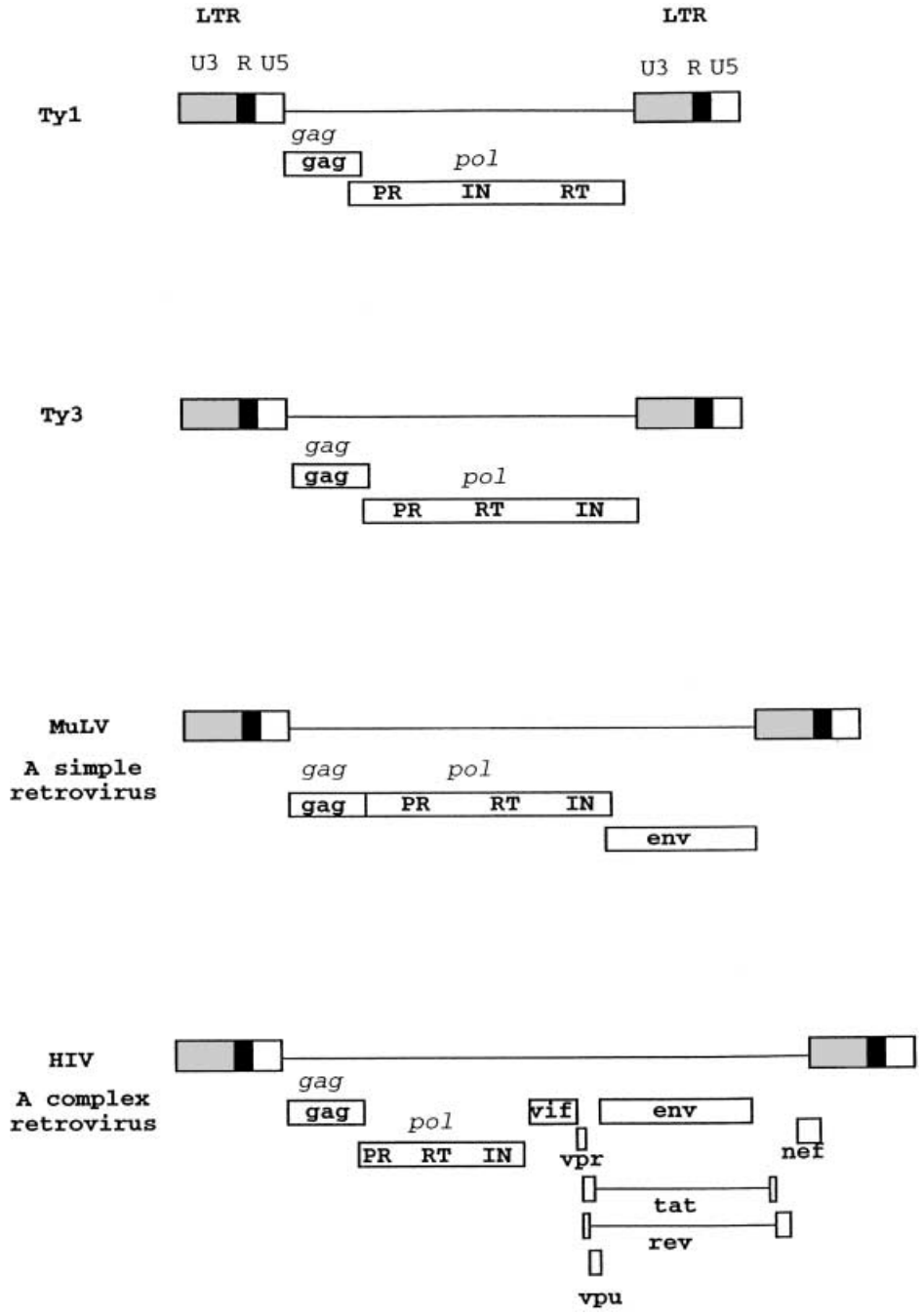

Figure 1. Genetic organization of prototypic retrotransposons and retroviruses. The scale of the long terminal repeats (LTRs) composed of U3, R and U5 elements has not been respected. Open rectangles indicate open reading frames. Rectangles offset vertically have different reading frames. In HIV the horizontal lines connecting two rectangles indicate splicing out of the segment between the two rectangles. Ty1 and Ty3 are two retrotransposons of the yeast *Saccharomyces cerevisiae;* MuLV, murine leukemia virus; HIV, human immunodeficiency virus.

of reverse transcription of LTR retrotransposons was learned from the work on the yeast Ty elements by identifying replication intermediates synthesized in the virus-like particles (VLPs) isolated from yeast cells overexpressing these elements [9–12].

The basic features of the process of replication of LTR retrotransposons are outlined in figure 2. RT plays a central role in this process. It is a multifunctional enzyme which possesses RNA-dependent and DNA-dependent DNA polymerase activities as well as RNase H activity that specifically degrades the RNA strand of RNA-DNA duplexes. At some stages of the replication a strand-displacement activity of RT is also necessary. All activities are essential for the conversion of single-stranded ge-

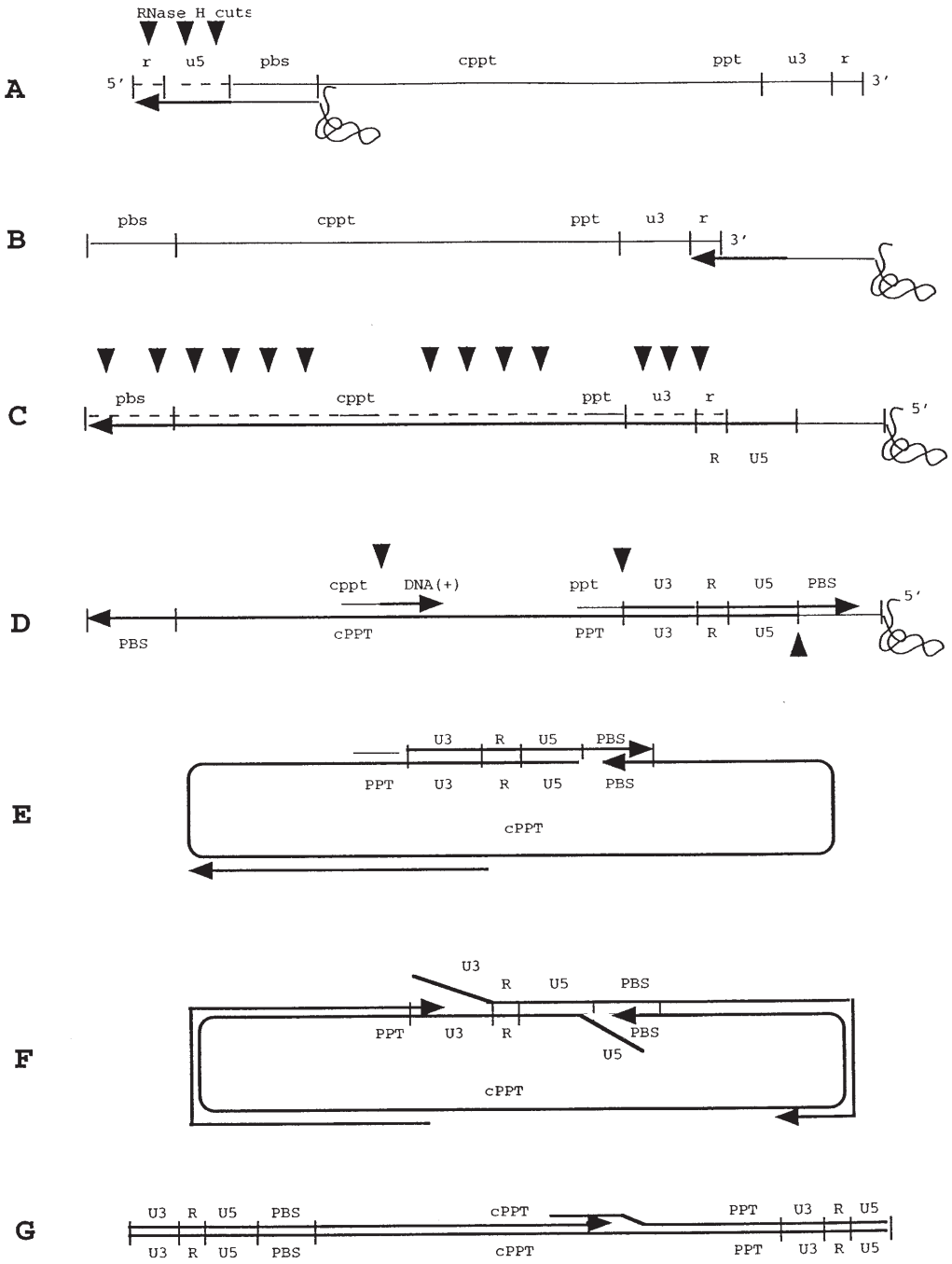

Figure 2. (adapted from Götte et al. [66]). Reverse transcription of retrotransposon or retroviral genomic RNA into double stranded preintegrative DNA. RNA is represented by lowercase letters and thin lines, whereas DNA is represented by capital letters and bold lines. Retroviruses and the Ty1 retrotransposon [94] have a diploid RNA genome. For clarity, only one RNA strand is shown. In theory both inter- and intramolecular transfer can occur during first strand (step A to B) or second strand transfer (step D to E). Several groups have demonstrated that the first strand transfer can be either inter- or intramolecular [95–98]. Most studies agree that the second strand transfer is almost always intramolecular, probably because only a single intact duplex DNA is synthesized in virions or VLPs. (*A*) Primer tRNA anneals to the primer binding site. −sssDNA is synthesized. The r and u5 regions of the RNA template are degraded by RNase H. (*B*) First strand tranfer: −sssDNA is translocated and annealed to the 3′ end of the genomic RNA. (*C*) Synthesis of minus-strand DNA resumes. The genomic RNA is further degraded by RNase H. Small polypurine rich fragments of RNA (PPT) remain undegraded. (*D*) Two preferred PPTs (the 3′ and central PPTs) serve as primers for initation of plus-strand synthesis. +sssDNA synthesis terminates when RT reaches the modified base nearest the primer tRNA 3′ terminus. The PPTs are cleaved by RNase H at the RNA-DNA junction. (*E*) Second strand transfer: +sssDNA anneals to the 3′ terminus of minus-strand DNA via PBS-PBS hybridization. (*F*) Reverse transcriptase completes plus-strand and minus-strand DNA synthesis. (*G*) The full-length retroelement DNA is completed. It is longer than the genomic RNA since reverse transcriptase has created duplications of the U5 and U3 regions at the ends of the DNA. As a consequence of initiation of plus-strand DNA synthesis from the central PPT, the preintegrative DNA displays a discontinuity in the plus strand.

nomic RNA into the double-stranded preintegrative DNA. This review focuses on the role of RT in the different steps of the replication process of retroelements. The features of retrotransposon replication which differ from the retroviral ones will be emphasized. The properties of retroviral RTs have been extensively studied in vitro. In contrast, until recently the RTs of retrotransposons have been studied only in the context of purified VLPs. Active recombinant RT of the two yeast retrotransposons Ty1 and Ty3 have now been obtained [13–15]. Given that it would be too extensive to discuss the large number of data on retroviral RTs, this review concentrates on in vitro properties of the two newly characterized retrotransposon RTs. Other reviews of retrotransposon and retroviral RTs can be found in two books and references therein [16–17].

Reverse transcription of retroelements

Annealing of the primer to the RNA template and initiation of minus-strand DNA synthesis

Primers used by RTs and primer binding sites

Like many other DNA polymerases, RT uses a primer carrying a free 3′-OH to initiate reverse transcription. All retroviruses and most retrotransposons utilize the 3′ end of a host-encoded transfer RNA (tRNA) to prime minus-strand complementary DNA (cDNA) synthesis (table 1) [18–22]. However, some retrotransposons do not utilize mature tRNA as primer. For example, the primers of the *Drosophila melanogaster copia* element [23] and of the *Saccharomyces cerevisiae* Ty5 element [24] are internal portions of a tRNA. Interestingly, both elements use a fragment of the host $tRNA_i^{Met}$ which is cleaved at the same position in the anticodon arm to generate the RT primer. The mechanism of cleavage of $tRNA_i^{Met}$ remains unknown. The suggestion that RNase P overprocessing could do the specific cleavage has not been verified [25]. Other novel priming mechanisms have been described. Hepatitis B virus initiates DNA synthesis from an OH group provided by a tyrosine residue of the RT [26]. The Tf1 retrotransposon from the yeast *Schizosaccharomyces pombe* uses a self-priming mechanism to initiate reverse transcription [27–29]. The first 11 bases at the 5′ end of the Tf1 template messenger RNA (mRNA) fold back and anneal to the primer binding site (PBS) sequence near the 5′ end of the mRNA. A cleavage between the 11th and 12th allows the first 11 ribonucleotides to prime reverse transcription.

The PBS of all retroelements is localized near the 5′ end of the plus-strand genomic RNA. For retroviruses it is complementary to 18 bases at the 3′ terminus of the primer tRNA, whereas it varies from 8 to 18 bases in retrotransposons (table 1). For several retroelements in vivo and in vitro studies support the existence of interactions between the primer tRNA and genomic RNA in regions other than the PBS sequence (fig. 3) [30–41]. These extended interactions help stabilize the primer-template complex and are probably necessary to form specific structures recognized by the cognate RT to initiate minus-strand DNA synthesis. To test this hypothesis Arts et al. [42] have evaluated the efficiency with which several lentiviral (HIV-1, FIV, EIAV, HIV-2, SIV) and retroviral (MLV, AMV) RTs support minus-strand DNA synthesis from $tRNA^{Lys}$ hybridized to the PBS-containing RNA template of HIV-1. With the exception of HIV-1, AMV and SIV RTs, no other enzyme initiated DNA synthesis from this primer template. Thus, although the lentiviral RTs tested in this study share a common $tRNA^{Lys}$ replication primer, this result suggests that efficient initiation of minus-strand DNA synthesis may be defined by retrovirus-specific interactions between the primer template and its cognate RT. In the yeast *S. cerevisiae* we have shown that disruption of some of the interactions of the Ty1 primer $tRNA_i^{Met}$ in regions outside of the PBS abolishes initiation of minus-strand DNA synthesis in vivo [35]. Gabus et al. [37] have shown that the yeast retrotransposon Ty3 has an unusual bipartite primer binding site with segments of complementarity to primer $tRNA_i^{Met}$ located to the opposite ends of the genome i.e.

Table 1. tRNA primers of selected retroviruses and retrotransposons. Human immunodeficiency virus-1 (HIV-1), human foamy virus (HFV), avian sarcoma leukosis virus (ASLV), murine leukemia virus (MuLV). *gypsy* and ZAM are two *Drosophila* retroviruses, CaMV is the cauliflower mosaic virus and Tnt1 is a tobacco retrotransposon.

Retroviruses	Primer	Length of PBS
HIV-1	3′ end of $tRNA_3^{Lys}$	18
HFV	3′ end of $tRNA^{Lys}$	18
ASLV	3′ end of $tRNA^{TrP}$	18
MuLV	3′ end of $tRNA^{Pro}$	18
gypsy	3′ end of $tRNA^{Lys}$	11
ZAM	3′ end of $tRNA_4^{Ser}$	12
S. cerevisiae retrotransposons		
Ty1/Ty2	3′ end of $tRNA_i^{Met}$	10
Ty3	3′ end of $tRNA_i^{Met}$	8
Ty4	3′ end of $tRNA^{Asn}$	18
Ty5	fragment from the 5′ region of $tRNA_i^{Met}$	14
Drosophila retrotransposons		
17, 6/297/tom	3′ end of $tRNA^{Ser}$	18
mdg1, 412	3′ end of $tRNA^{Arg}$	18
copia	fragment from the 5′ region of $tRNA_i^{Met}$	14
Pararetrovirus		
CaMV	3′ end of $tRNA_i^{Met}$	13
Plant retrotransposon		
Tnt1	3′ end of $tRNA_i^{Met}$	12

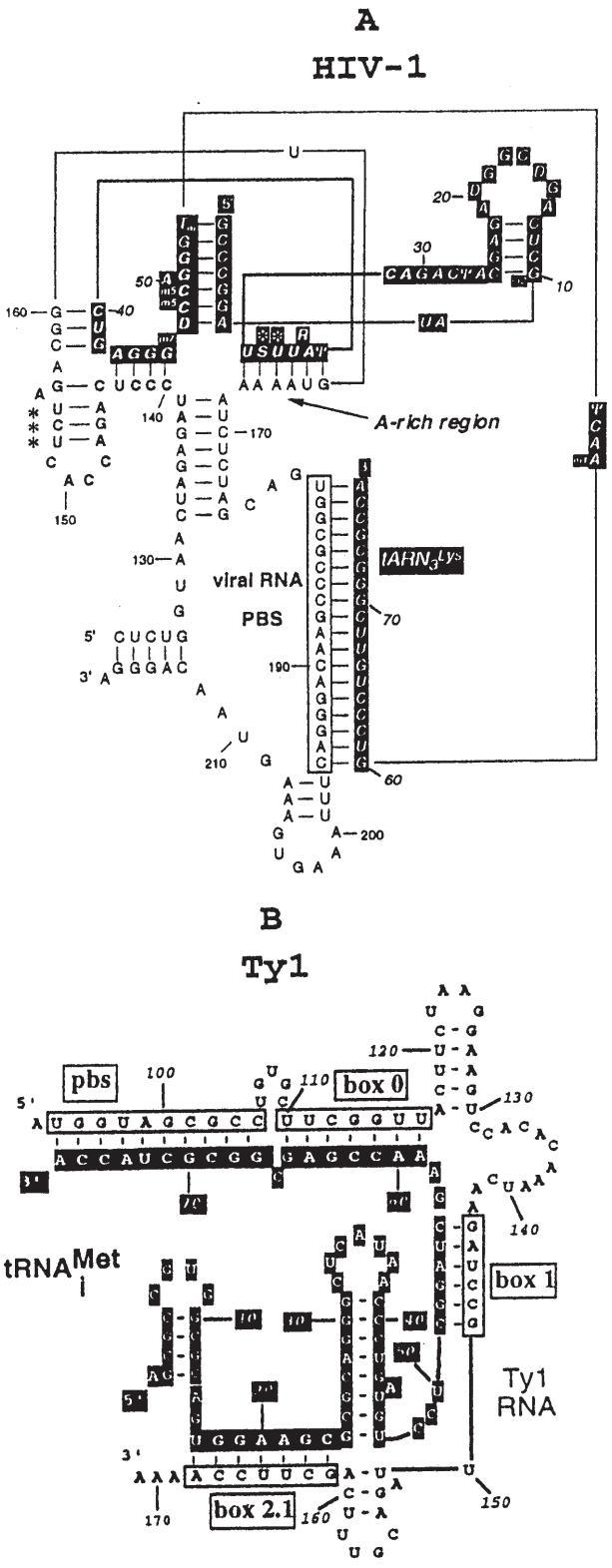

Figure 3. Extended primer-template interactions in HIV-1 and Ty1. (*A*) Secondary structure of HIV-1 primer-template [32]. The pbs is boxed. $tRNA^{Lys}$ is in white letters on a black background. The A-rich region important for HIV-1 replication [39–40] is indicated by an arrow. (*B*) Secondary structure of the Ty1 primer-template [34]. The pbs is boxed. $tRNA_i^{Met}$ is in white letters on a black background. Mutations in boxes 0, 1 and 2.1 affect initiation of DNA synthesis [35].

a region of 8 nucleotides located adjacent to the 5′ LTR and two adjacent sequences of 12 and 11 nucleotides located near the 3′ end of the Ty3 genomic RNA. Each of these complementary segments is essential for efficient initiation of Ty3 reverse transcription. These results suggest that interactions outside the PBS are probably necessary to optimize the fit between the active site of the enzyme and the primer template in order to allow initiation of reverse transcription.

Annealing of the primer to the template

Since RNA molecules possess ordered secondary and tertiary structures, annealing of the tRNA primer to the genomic RNA template cannot occur without the help of cofactors to unwind the RNA molecules. In vitro results suggest that interactions of RT with the primer tRNA could induce a destabilization of the structure of tRNA and facilitate unwinding of the tRNA [19, 22]. However, other experiments show that annealing of the tRNA onto the PBS cannot be done by RT alone, implying that additional factors are needed for the formation of the primer-template initiation complex [43]. Such a factor is the NC protein, which possesses the capacity to unwind nucleic acids and promote annealing of the tRNA to the PBS of the RNA template [44–49]. It has been proposed that interactions between the RT and NC are instrumental for formation of the initiation complex and for efficient reverse transcription of the RNA template.

Synthesis of minus-strand strong-stop DNA

Once the primer template has been formed, RT initiates reverse transcription by extending the 3′ end of the primer which uses the 5′protruding end of the plus-strand genomic RNA as template. When RT reaches the 5′ end of the RNA template, the so-called minus-strand strong-stop DNA (–sssDNA) is released and transferred to the 3′ end of the genomic RNA in order to allow continuation of minus-strand DNA synthesis (fig. 2B). During minus-strand DNA synthesis, the template RNA strand of the newly formed RNA-DNA hybrid is degraded by the RT-associated RNase H activity [50–53]. Two modes of RNase H activities can be distinguished, (i) a polymerase-dependent mode which accompanies DNA synthesis and is directed by the polymerase domain binding to the recessed nascent DNA 3′ end and (ii) a polymerase-independent mode which occurs without DNA synthesis but is positioned by the polymerase domain binding to the recessed 5′ end of the RNA paired to the DNA. The polymerase-dependent RNase H activity which takes place during minus-strand synthesis is not sufficient to completely degrade all of the template RNA. The main reason for this partial digestion is that the rate of polymerization is greater than the rate of RNA template cleavage [54].The fragments which remain bound to the minus-strand DNA must be digested further by the polymerase-independent activity of RNase H

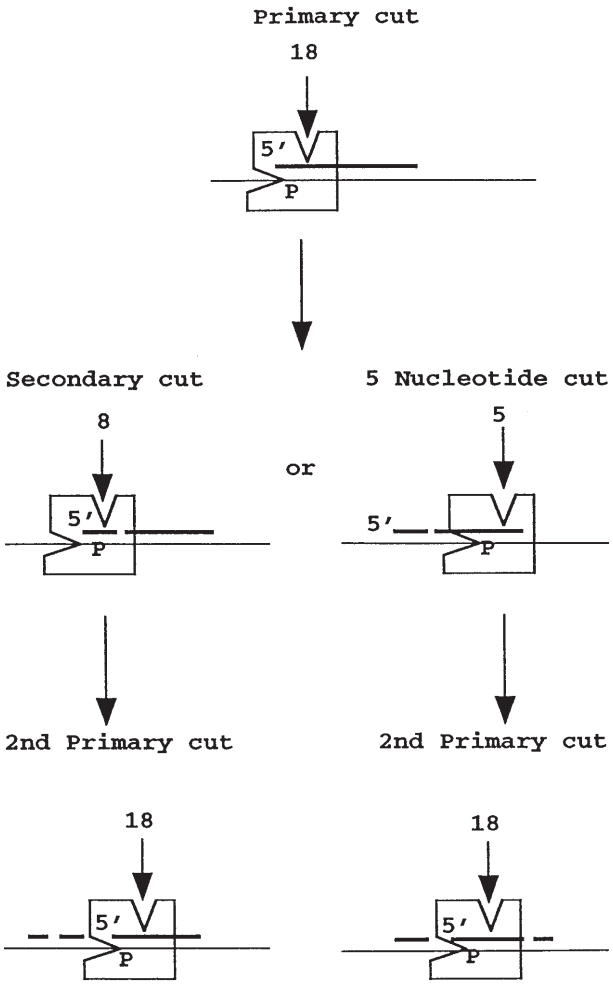

Figure 4. (adapted from Wisniewski et al. [51]). Progressive cleavage mechanism of RNase H. RNA is represented by bold lines, DNA is represented by thin lines. A primary cut is made by the RNase H active site positioned 18 nt from the 5′ end of the RNA. The enzyme rebinds or slides toward the 5′ end of the RNA to make a secondary cut 8 nt from the 5′ end of the RNA. RT can also align the RNase H active site to the 3′ end of the RNA, cutting five residues in. RT then binds to the new 5′ end of the RNA created by the first primary cut or the secondary cut to make a second primary cut.

through a complex series of steps (fig. 4). The RNA fragments which are not removed after RNase H digestion are eventually displaced by the polymerase during the subsequent synthesis of plus-strand DNA.

First strand transfer

The strand transfer steps in the model of retroviral and retrotransposon DNA replication explains how synthesis which initiates near the 5′ end of the RNA genome can generate an intact preintegrative DNA with two LTRs. The first strand transfer occurs when the completed –sssDNA which has been cleared from the template RNA by RNase H is transferred from the 5′ end of the genomic RNA to its 3′ end (fig. 2B). This is possible because the terminal region of the –sssDNA is complementary to the r region at the 3′ end of the template RNA. The complementary sequences of minus-strand DNA and plus-strand RNA anneal, thereby allowing DNA synthesis to resume. It is not known whether strand transfer is driven only by base pairing between the complementary regions of minus-strand DNA and plus-strand RNA or whether cofactors are involved to help annealing. In vitro results have shown that RT remains bound to the newly synthezised –sssDNA and that it can catalyze DNA strand transfer reaction alone [55]. Thus. RT may play an active role in the process of strand transfer by stabilizing the interaction between the –sssDNA strand and the complementary region of the acceptor template. Bearing this in mind, a model of first strand transfer proceeding through an RNA-DNA-RNA intermediate accomodated by RT has been proposed for HIV-1 by Peliska and Benkovic (fig. 5) [55]. Xu and Boeke [56] have proposed a 'search and jump' model for template switching during reverse transcription of the yeast Ty1 element. They suggest that RT polymerizes DNA on the RNA template in a series of runs and pauses. During the pauses, the template and nascent DNA may become separated and reannealed to other complementary sequences. This model, which explains observations that tandem repeats in yeast Ty1 element are deleted during reverse transcription, can be extended to first strand transfer: after completion of –sssDNA synthesis, RT pauses at the end of the template; the –sssDNA complementary to the r sequence at the 5′ end of the genomic RNA is then separated from the template RNA which has not been digested by RNase H and anneals to the repeated r sequence at the 3′ end of the genomic RNA.

Synthesis of plus-strand DNA

Generation of plus-strand primer

After first strand transfer, synthesis of minus-strand continues until RT reaches the 5′ end of the RNA genome (fig. 2C). As synthesis of minus-strand DNA proceeds, the RNA is digested by RNase H. However, specific purine-rich fragments called polypurine tracts (PPTs) which are resistant to RNase H digestion function as RNA primers for plus-strand DNA synthesis (table 2) [50]. In all retroelements a preferred PPT is located just upstream of the 3′ LTR boundary. Cleavage at the 5′ and 3′ ends of the PPT must be specific in order to generate functional plus-strand primers. Particularly, the precision of plus-strand initiation from the 3′ end of this PPT is important because it defines the 5′ border of the upstream LTR which contains cis-acting sequences required for integration of the preintegrative DNA into the host cell genome. In most retroelements plus-strand DNA synthesis is also initiated at other sites. Several lentiviruses (including HIV-1) and the yeast Ty1 element use a second

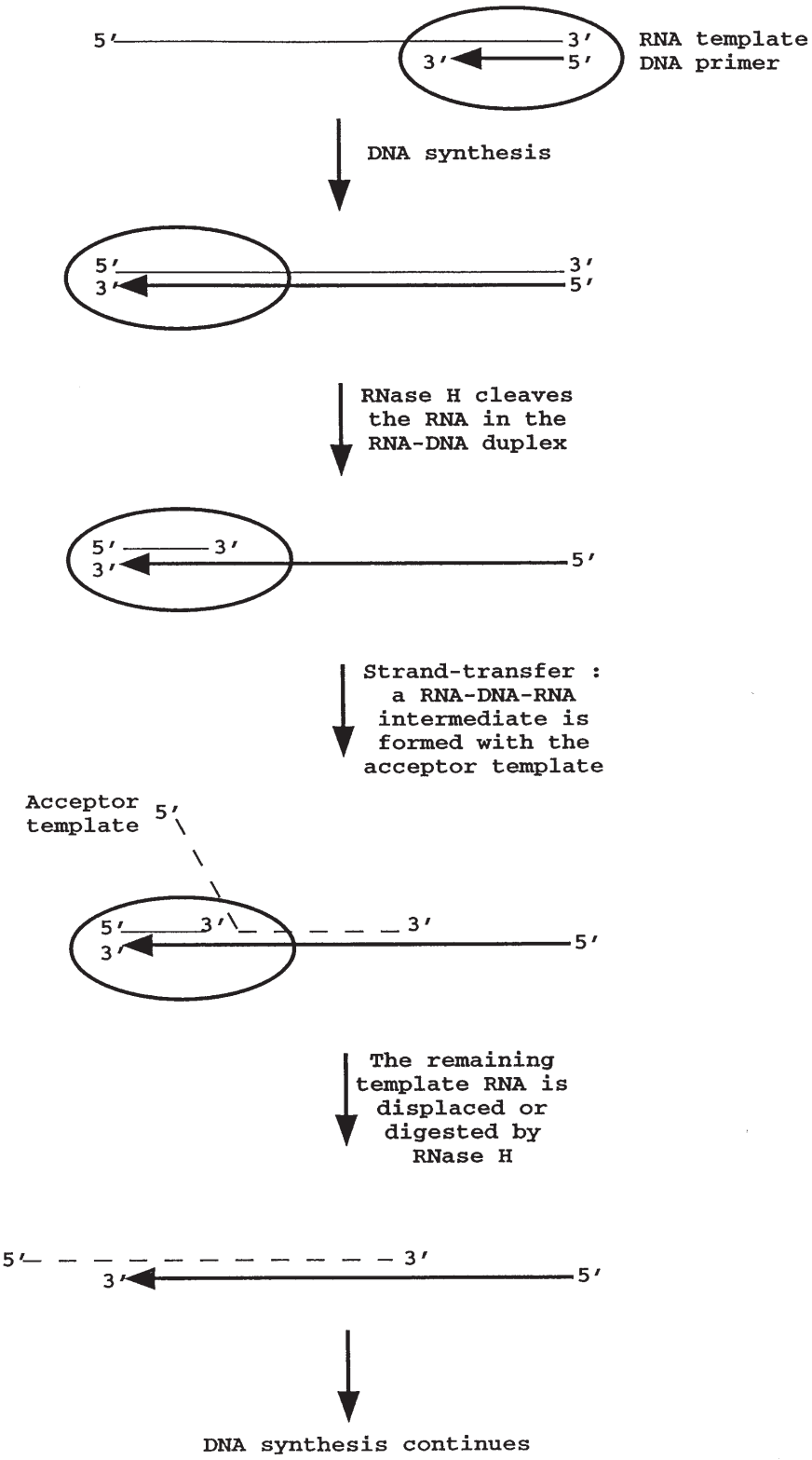

Figure 5. (adapted from Peliska and Benkovic [55]). Model for DNA strand transfer proceeding through an RNA-DNA-RNA intermediate accomodated by RT. RNA is represented by thin lines, DNA is represented by bold lines.

Table 2. Polypurine tracts and U boxes of selected retroviruses and retrotransposons. U-rich sequences upstream of the PPT are shown in bold face. PPT sequences are underlined. No U-box can be identified upstream of the PPT of CAEV or Ty3. Sequences are from human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), mouse intracisternal A-Particle (IAP), mouse mammary tumor virus (MMTV), Moloney murine leukemia virus (Mo-MuLV), avian leukosis virus (ALV), Mason-Pfizer monkey virus (MPMV), human endogenous retrovirus (HERV-K10), cauliflower mosaic virus (CaMV), caprine arthritis-encephalitis virus (CAEV), and yeast retrotransposons Ty1 and Ty3.

	Genus	PPT
HIV-1 (NL43)	lentivirus	CAC**UUUUU**AAAAGAAAAGGGGGGACUGGA
HIV-1 (LAI)	lentivirus	CAC**UUUUU**AAAAGAAAAGGGGGGACUGGA
HIV-2 (BEN)	lentivirus	ACA**UUUUAU**AAAAGAAAAAGGGGGACUGCA
SIV (mac239)	lentivirus	UCA**UUUUAU**AAAAGAAAAGGGGGGACUGGA
IAP	Type A oncovirus	GCC**UAUUUGCUCUUAUU**AAAAGAAAAAGGGGGAGAUGUU
MMTV	Type B oncovirus	UGC**UUCUUUU**AAAAAAGAAAAAAGGGGGAAAUGCC
Mo-MuLV	Type C oncovirus	AGA**UUUUAUUUAGUCU**CCAGAAAAAGGGGGGAAUGAA
ALV	Type C oncovirus	CGC**UUUU**GCAUAGGGAGGGGGAAAUGUA
MPMV	Type D oncovirus	GUC**UUUUGUUUUUUAUAAUU**AAAAAGGGUGACAUGUC
HERV-K10	endogenous retrovirus	CGG**UUUUGU**CGAAAAGAAAAGGGGGAAAUGUG
CaMV	plant pararetrovirus	CCA**UUUUU**AAGAGUGGGGGGGUUGAU
Ty1	retrotransposon	AUC**UAUUACAUUAU**GGGUGGUAUGUU
CAEV	lentivirus	CUUGGAGAACACCACAAAAAUAAAAAAAAGAAAGGGUGAC
Ty3	retrotransposon	UACGAAACACAAGACAACCCUGAGAGAGAGGAAGAUGUU

preferred initiation site near the center of the genome [57–59]. A consequence of DNA plus-strand synthesis from internal sites is that linear preintegration DNA molecules have an interrupted plus strand. In HIV-1, a central strand-displacement event consecutive to central initiation and termination of plus-strand synthesis creates a plus-strand overlap. Zennou et al. [60] have provided evidence for the role of this central DNA flap in HIV-1 nuclear import. Contrary to HIV-1, the second plus-strand priming site of Ty1 appears to be dispensable for replication and transposition.

The mechanism by which RNase H creates the PPT primer is not well understood. Several reports have suggested that the precise generation of PPT primers is sequence dependent, i.e. the integrity of some positions in the PPT is necessary for cleavage specificity [50]. Recently, a conserved U-stretch within the upstream sequence of several retroelements (table 2) was found to be important for their replication [61–65]. In Ty1 it has been shown that mutations within this U-rich region inhibit plus-strand strong-stop DNA synthesis (+sssDNA) [64]. Robson and Telesnitsky [63] propose that contacts between the DNA polymerase domain of RT and the U-rich sequence upstream of the PPT during DNA polymerization might contribute to the specificity of the RNase H cleavage at the PPT.

Polypurine tract primer generation by MoMLV RT has been studied by Schultz et al. [53] with model hybrid substrates representing various primer-template possibilities that might occur at or near the beginning of plus-strand DNA synthesis. By using a long 5′ end-labelled RNA-DNA duplex containing the PPT, an internal cleavage producing the 3′ end of the PPT primer was observed. This suggests that neither the polymerase-dependent nor the 5′-end-directed cleavage mechanism of RNAse H are necessarily required to generate the 3′ end of the plus-strand primer, and that the PPT sequence alone or the structure of the RNA-DNA duplex [66] containing the PPT might be sufficient to define the cleavage specificity at the 3′ end of the PPT primer.

Synthesis of plus-strand strong-stop DNA and second strand transfer

Once the PPT primer is generated, plus-strand synthesis is initiated using the 5′ end of minus-strand DNA and a portion of the tRNA attached to the minus-strand DNA as template (fig. 2D). Plus-strand strong-stop DNA terminates when RT reaches the modified base nearest the primer tRNA 3′ terminus. At this stage the primer tRNA attached to the minus-strand DNA is removed by RNase H. In the model for retroviral reverse transcription, the +sssDNA is transferred to the 5′ end of the RNA (second strand transfer, fig. 2E). The plus-strand PBS sequence, templated by the tRNA, pairs with the minus-strand complementary sequence. The prediction of this mode of second-strand transfer is that the primer tRNA sequence is inherited during replication of retroelements. This seems to be the case for retroviruses since experiments with mutants in which the PBS sequence of the genomic RNA has been mutated or deleted demonstrate that wild-type PBS is regenerated after several passages [41]. Surprisingly, Lauermann and Boeke [67–69] have shown that in Ty1, PBS mutants are not healed by the wild-type tRNA sequence. Thus, in Ty1, the primer binding site sequence is not inherited from the tRNA as in retroviruses. To explain this result a plus-strand primer recycling model was proposed (fig. 6). Analysis of the Ty3 element,

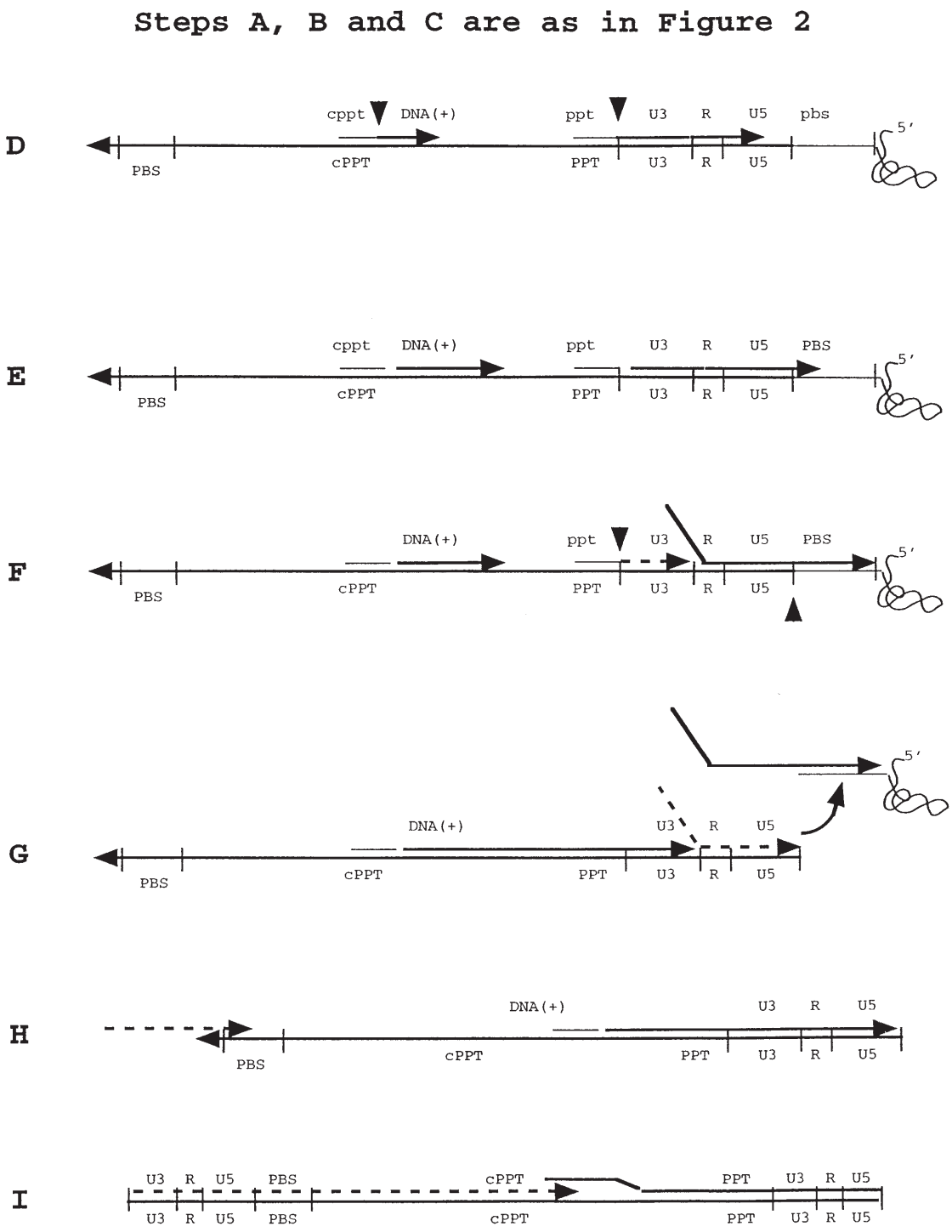

Figure 6. (adapted from Lauermann and Boeke [69]). Recycling model for Ty1 reverse transcription. Steps *A*, *B* and *C* are as in figure 2. (*D*, *E*) Two preferred PPTs (the 3′ and central PPTs) serve as primers for initiation of plus-strand synthesis. The PPTs are cleaved by RNase H at the RNA-DNA junction. (*F*) The 3′ PPT is recycled, a second round of +sssDNA synthesis is initiated. The second +sssDNA begins to displace the first one. (*G*) The first +sssDNA is removed; it is probably a dead-end product. The second +sssDNA begins to be displaced by the plus-strand DNA initiated at the central PPT. (*H*) Second strand transfer: +sssDNA anneals to the 3′ terminus of minus-strand via complementarity to a portion of R-U5 that remains upstream of the PBS following unprecise RNase H cleavage [99, 100]. (*I*) Reverse transcriptase completes plus- and minus-strand DNA synthesis.

evolutionarily distant from Ty1, indicates that Ty3 shares the plus-strand strong-stop DNA transfer strategy with Ty1.

Completion of plus- and minus-strand DNA syntheses (fig. 2F and G)

After second-strand transfer has occurred, RT continues to elongate the plus-strand DNA until it reaches the second plus-strand DNA initiated at the central PPT. Plus-strand synthesis stops after a small fragment of the second plus-strand DNA has been displaced, thus creating a plus-strand DNA overlap. Concomitently, RT extends and completes minus-strand synthesis by using the 5′ end of plus strand DNA as a template. The minus-strand DNA from which the plus-strand was copied must be displaced by the strand-displacement activity of RT (possibly with the help of nucleocapsid protein). The final product is a double-stranded DNA with a long terminal repeat at each terminus.

RTs of the yeast retrotransposons Ty1 and Ty3

RTs of retrotransposons have been expressed as recombinant proteins only recently [13–15]. Thus, contrary to retroviral RTs, their properties have not been studied extensively in vitro. However, it has been known for a long time that RT activity can be detected in yeast cells overproducing Ty1 and Ty3 elements and that it is associated with virus-like particles (VLPs) that contain Ty RNA and DNA [6–8]. Unfortunately, it has not been possible to use VLP preparations as a source of active purified RT. In order to be able to do structural and enzymatic studies of LTR retrotransposon RTs, there was a need to express active RT in bacteria using recombinant DNA methodology. In the Ty1 and Ty3 elements, open reading frames with amino acid homology to other RTs are encoded by the *pol* gene (fig. 7). The primary gene product of *pol* is synthesized as a Gag-Pol fusion protein involving a +1 frameshift to put the *gag* gene in frame with *pol*. The precursor Gag-Pol protein is then processed by the retrotransposon-encoded protease. On the basis of pulse-chase experiments and immunoblot analyses of Ty1 proteins, Garfinkel et al. [70] have proposed the following processing pathway of the Ty1 Gag-Pol polyprotein: the 199-kDa Gag1-Pol1 polyprotein is cleaved to form the Gag1 protein and a PR-IN-RT 160-kDa processing intermediate which is further processed into mature PR and p140. The p140 intermediate is finally cleaved to form mature IN and RT. By determining the role of individual proteolytic cleavage sites between Gag1 and PR, PR and IN and IN and RT, Merkulov et al. [71] have provided evidence for a semiordered processing pathway of Ty1 polypeptides in agreement with the results of Garfinkel et al. [70], i.e. the Gag1-PR cleavage site is cleaved first, and its cleavage is required for subsequent processing of the PR-IN and IN-RT sites. Thus, the final products of Gag1-Pol1-p199 kDa are four mature proteins: CA (Gag-p45), PR (Pol-p20), IN (Pol-p71) and RT (Pol-p63) (the names in parentheses are the new names of the Ty1-encoded proteins proposed by Merkulov et al. [71] to reflect the fact that the masses of all Ty1 peptides are known and to conform with retroviral standards). In Ty3, the gene product of *pol* is also synthesized as a Gag3-Pol3 multiprotein which is processed into five mature proteins homologous to retroviral CA, PR, RT, IN and nucleocapsid (NC) [72]. A 10-kDa domain (the J domain) between PR and RT, dispensable for Ty3 transposition, has been recently characterized by Claypool et al. [73]. Sequence alignement, mutagenesis, immunoblot analysis and amino-terminal sequence determination has made it possible to identify the Ty3 RT as a 55-kDa protein.

Using the polymerase chain reaction, the RT open reading frames were amplified from the pol gene of Ty1 and Ty3, expressed in *E. coli* and the enzymatic properties of the recombinant proteins were characterized [13–15].

Ty3 RT

Biochemical properties of the recombinant Ty3 RT.

Recombinant Ty3 RT was expressed in *E. coli* as a 55-kDa protein containing a short polyhistidine extension at its N-terminus [13, 14]. It was purified to near homogeneity by a combination of metal chelate and ion-exchange chromatography. Results of size-exclusion chromatography indicate that Ty3 RT has a monomeric organization. In contrast to most lentiviral RTs, which exhibit a dimeric structure, Ty3 RT might be active as a monomer like the recombinant enzymes from BLV [74], MLV [75] or MMTV [76].

The enzymatic activity of the purified protein was measured with distinct template primers. Using the synthetic poly(rA):oligodT template-primer system, Ty3 RT was found to be as active as the retroviral HIV-1 or MLV enzymes and to polymerize polydT products of identical size. DNA-dependent DNA polymerase activity of Ty3 RT was evaluated on a 71nt DNA template-36nt DNA primer used previously by Le Grice et al. to characterize many retroviral RTs [77–78]. The capacity of this substrate to form intramolecular base pairing (hairpin) at the 5′ end of the template was exploited to evaluate the processivity of RTs. The response of Ty3 RT to this structure showed that its polymerase activity was affected both by the template hairpin and by temperature. The Ty3 enzyme has a low temperature optimum (30 °C), which can be related to the temperature sensitivity of the in vivo transposition process. At 37 °C the Ty3 RT is stopped by the hairpin near the 5′ end of the template, whereas it has the capacity to polymerize through the hairpin at 30 °C.

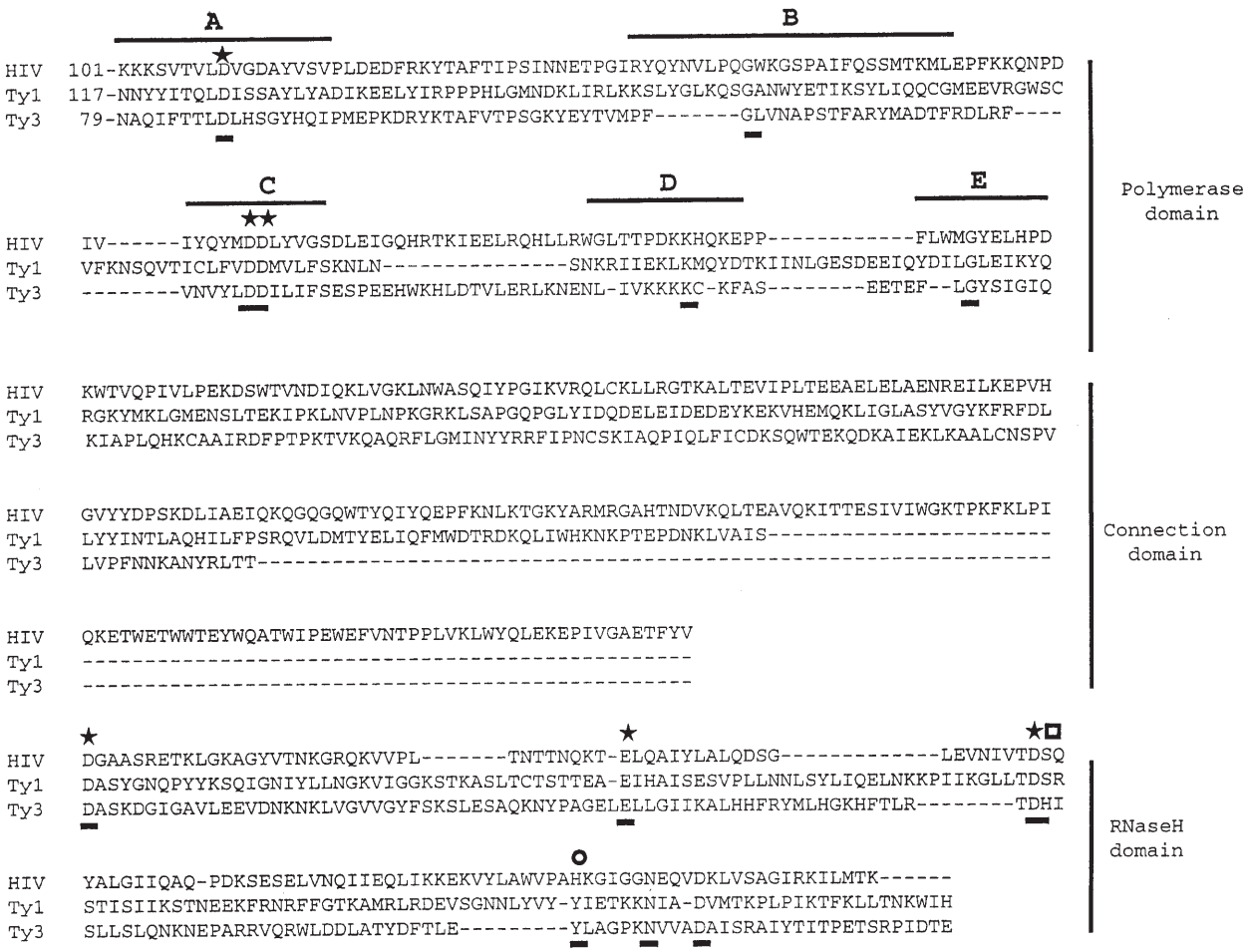

Figure 7. Sequence alignment of Ty1, Ty3 and HIV-1 RTs. Three domains have been identified by vertical lines: the polymerase domain, the connection domain and the RNase H domain. (i) Polymerase domain: alignment of the five conserved motifs (*A* to *E*, indicated by black bars above the alignment) within the polymerase domain was as described by Poch et al. [1]. Motif C of Ty3 and HIV-1 contains the highly conserved sequences YLDD and YMDD characteristic of reverse transcriptases. Ty1 has the related sequence FVDD. The nearly invariant amino acid residues found in the polymerase domain of all RNA-dependent polymerases are underlined. The three catalytically essentiel D residues are highlighted by stars. (ii) The connection domain extends from the last residue in the conserved motif E to the first invariant residue of the RNase H domain [92]. The connection domains of Ty1, Ty3 and HIV-1 contain 133, 92 and 205 residues, respectively. (iii) RNase H domain: alignment of the RNase H domains was as described by McClure [92]. The catalytically essentiel residues are indicated by stars. The nearly invariant amino acid residues found in all RNase H domains are underlined. In HIV-1 RT the H residue indicated by an open circle serves to activate a water molecule in the active site of the enzyme. In Ty1, Ty3 and the *gypsy* group of retroelements this H residue is substituted with an invariant Y residue. In Ty3 and in the *gypsy* group (but not in Ty1) a highly conserved H residue (open square) is found immediately adjacent to one of the D residue of the active site. Rausch et al. [14] propose that the H residue of the adjacent DH pair serves to activate a water molecule in the same manner as the H residue of HIV-1. The numbers at the beginning of the sequences indicate the number of residues from the N-terminus of the RTs omitted from the figure.

Compared with HIV-1 RT, the Ty3 enzyme seems to have a more robust strand-displacement activity.

The C-terminal portion of Ty3 RT encodes a functional RNase H domain. Enzymatic footprinting and RNase H hydrolysis of replication complexes support the notion that the spatial separation of the polymerase and RNase H catalytic center of Ty3 RT exceeds the 18 bp observed with most retroviral enzymes. Rausch et al. [14] speculate that the increased spacial separation between the catalytic center could be related to the longer bipartite Ty3 PBS contributed from both ends of the genome.

To determine whether the recombinant Ty3 protein was able to catalyze more specialized activities, Rausch et al. [14] investigated the recognition of both PPT and non-PPT-containing RNA primers by the Ty3 enzyme using a PPT-scanning strategy. Three plus-strand RNA primers 25-nt in length (P1, P2 and P3) were hybridized to different regions of a 65-nt minus-strand DNA oligonucleotide containing the PPT sequence. Primer P3 was complementary to the DNA sequence immediately 5′ to the PPT; P2 contained the PPT sequence and additional sequences 5′ to the PPT; and P1 contained the PPT sequence and ad-

ditional 3′ sequences. The different primer templates were incubated with the Ty3 RT and a deoxyribonucleotide triphosphate (dNTP) mixture to reveal the steps of primer selection and extension. The results of this experiment indicate that the non-PPT RNA primer P3 does not support plus-strand DNA synthesis. P1 and P2 support plus-strand DNA synthesis with different specificity and efficiency. Primer P1 must be first processed via RNase H cleavage at the PPT 3′ terminus before extension can occur. Analysis of the extension products generated from P1 indicates that the RNase H activity of Ty3 RT removes the RNA at the RNA-DNA junction during extension. Primer P2 is used most efficiently. The fact that no RNase H processing is required for initiation from this primer could explain its efficient utilization. These results indicate that purified recombinant Ty3 RT is able to recognize a polypurine tract primer from within a RNA-DNA hybrid, extend it into plus-strand DNA and excise the primer from nascent DNA.

NC-mediated initiation of Ty3 reverse transcription

The nucleocapsid proteins (NCps) are small basic proteins encoded by lenti- and oncoviruses which are required for virion structure and replication [44–45]. NCps contain one or two zinc fingers flanked by basic residues. They have RNA-DNA binding and annealing activities that play a role in the specificity of reverse transcription initiation and in the DNA strand transfers. The yeast Ty3 retrotransposon encodes NCp9, which possesses a unique zinc finger flanked by a long N-terminal domain and a short C-terminal domain. NCp9 is required for Ty3 transposition in yeast [37]. The role of NCp9 in primer tRNA-directed cDNA synthesis by Ty3 RT was examined by Cristofari et al. [13]. The Ty3 nucleocapsid was able to generate nucleoprotein complexes between the primer $tRNA_i^{Met}$ and Ty3 RNA and to induce a high level of DNA synthesis by the Ty3 RT. In the absence of NC, DNA synthesis was reduced to a very low level. Analysis of deletion mutants of NCp9 showed that the N-terminus but not the zinc finger is required for tRNA annealing to the PBS and for primer tRNA-directed cDNA synthesis by Ty3 RT. Thus, NCp9 chaperones bona fide cDNA synthesis by RT in the yeast Ty3 retrotransposon, as is the case for NCp7 in the HIV-1 system. The observation that Ty3 NCp9 and HIV-1 NCp7 are interchangeable using Ty3 and HIV-1 template-primer systems reinforces the notion that the two NCs have similar roles in the replication of Ty3 and HIV-1.

Ty1 RT

Expression of an active recombinant Ty1 RT

Mature RT transcriptase identified in Ty1 VLPs is a 63-kDa protein [70–71]. The 5′ and 3′ ends of the RT coding region can be deduced from the mass of the protein and from the N-terminal sequence reported by Moore and Garfinkel [79]. Thus, recombinant Ty1 RT was expressed in *E. coli* as a 63-kDa protein containing a short polyhistidine extension at its N-terminus. Surprisingly, the purified protein did not show any polymerase activity.

Several reports have implicated a role of the integrase domain in the enzymatic activity of some retroelement RTs [80–83]. We therefore constructed three IN-RT fusion recombinant proteins containing fragments of the C-terminal region of the Ty1 IN of increasing length (26, 115 and 145 residues) attached to the N-terminus of the RT domain. When the three IN-RT fusion proteins were expressed in *E. coli* and assayed for polymerase activity, only constructs with 115 and 145 residues of IN showed high DNA polymerase activity. The construct with 26 residues of IN was inactive.

The polymerase activity of recombinant enzyme with 115 residues of IN was tested by following the poly(rC):oligo(dG)-directed incorporation of α^{32}PdGTP. The recombinant enzyme had an absolute requirement for a divalent cation and was inhibited by increasing concentrations of NaCl. Maximal activity was observed at 20 mM Mg^{2+} and low NaCl concentration (15 mM). The recombinant enzyme was also active with Mn^{2+}, but like other RTs it showed a preference for Mg^{2+} over Mn^{2+}. In common with Ty3, the Ty1 RT had a temperature optimum between 15 °C and 25 °C. As noted for Ty3, this low-temperature optimum can probably be correlated to the temperature sensitivity of the Ty1 transposition process.

RTs are known to be sensitive to dideoxyribonucleoside triphosphates (ddNTPs), which act as chain terminators. The effect of increasing concentrations of ddGTP on poly(rC):oligo(dG)-directed polymerization of α^{32}PdGTP demonstrated that ddGTP is a strong inhibitor of Ty1 RT. Therefore, the behavior of Ty1 RT is similar to RTs from other eukaryotes which are inhibited by low ddGTP concentration.

RNase H activity of the recombinant Ty1 IN-RT enzyme

The RNase H activity of Ty1 enzyme was examined by following the cleavage of distinct RNA-DNA duplexes. Analysis of the RNA cleavage products on a denaturing gel showed that efficient cleavage was obtained with the recombinant Ty1 IN-RT enzyme.

By using a 35-nt RNA template – 28-nt DNA primer as substrate (fig. 8), the interdependence between the RNase H and polymerase active sites of the Ty1 enzyme could be analyzed. It was shown that the RNase H cleavage of the Ty1 enzyme was specific for the RNA phosphodiester bond between the 14^{th} and 15^{th} nucleotides downstream from the nucleotide complementary to the 3′ terminal nucleotide of the DNA primer. In comparison, HIV-1 en-

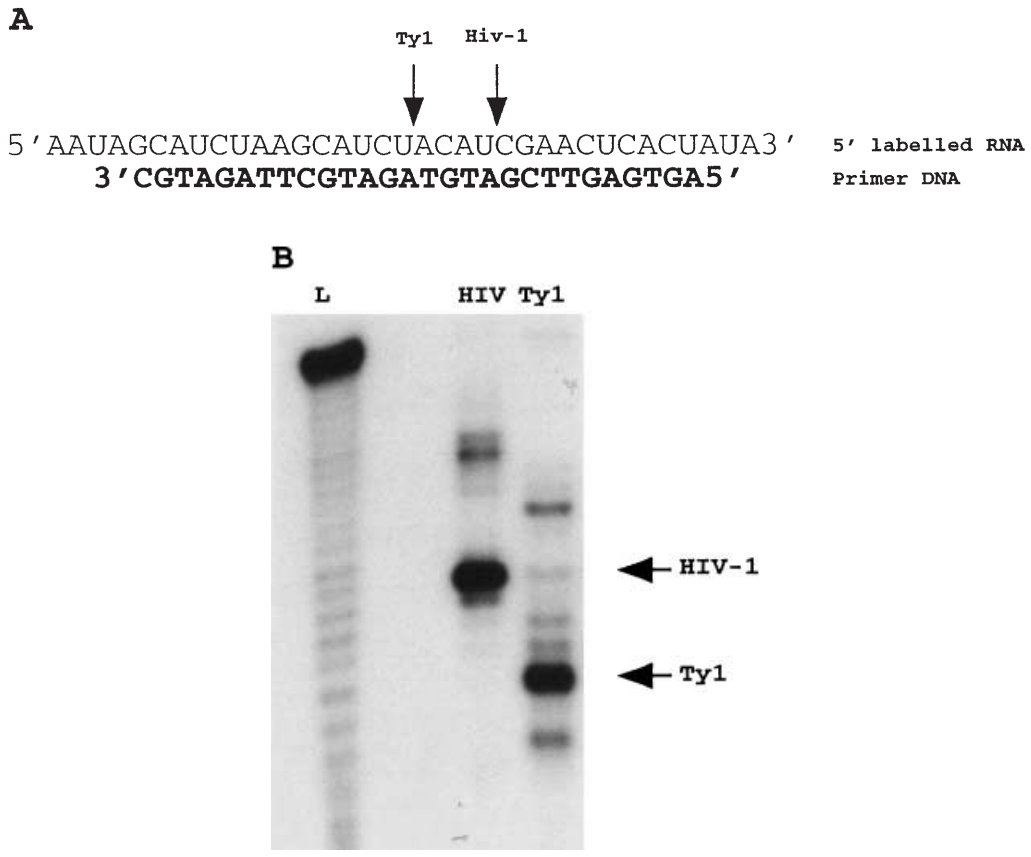

Figure 8. Interplay between the RNase H and polymerase active sites of Ty1 and HIV-1 RTs. A 35-nt-long RNA was annealed with a 28-nt-long complementary DNA primer; the DNA primer is shown in bold letters (*A*). The RNA template was radiolabelled at the 5′ end. The RNA-DNA heteroduplex and the Ty1 or HIV-1 enzyme were incubated to induce RNase H cleavage. The cleavage products analyzed on a 15% denaturing polyacrylamide gel are shown in *B*: lane L, alcaline hydrolysis of the RNA template; lane HIV-1 or Ty1, cleavage products of the RNA-DNA heteroduplex by HIV-1 or Ty1 RTs. The main cleavage product of Ty1 is 4 nt shorter than the main cleavage product of HIV-1.

zyme cleaved the RNA between positions 18 and 19, in keeping with biochemical and crystallographic data indicating that in HIV-1 RT the distance between the positions of RNase H cleavage and DNA polymerase active sites is 18–20 bp [54, 84–91]. Thus, the distance between the RNase H and polymerase active sites of the Ty1 enzyme is four nucleotides shorter than the distance measured for HIV-1 RT. Boeke [in 17] has speculated that the short (10 bases) PBS length might be correlated to a shorter distance between the two active sites in Ty1 compared with HIV-1. We suggest that this difference can be explained by a difference in structure of the two enzymes. The crystal structure of the p66 subunit of HIV-1 RT has shown that the polymerase domain is separated from the RNase H domain by a connection subdomain which forms a groove between the two active sites and plays a role in binding of the primer template [86]. Sequence comparison of Ty1 and HIV-1 RTs [1, 2, 92] shows that the connection domain of the Ty1 enzyme is shorter than the connection domain of the HIV-1 RT (fig. 7). This difference could explain the difference of length of primer template which can be accommodated between the polymerase and RNase H active sites of the two enzymes.

Concluding remarks

Replication of retrotransposons and retroviruses requires an RT capable of reverse-transcribing the RNA genomes into double-stranded DNA. The two active recombinant yeast Ty1 and Ty3 RTs which have been characterized will allow a more detailed analysis of the mechanism of retrotransposon replication at the biochemical level. The observation that an active Ty1 recombinant protein could be obtained only after adding amino acid residues encoded by the IN gene to the N-terminus of the RT domain suggests that interaction between the C-terminal region of the IN and the RT domain might be important for the function of the Ty1 enzyme. The question can be asked whether interactions between the IN and RT domains are necessary for the function of Ty1 RT in vivo. It has been previously reported that a 140-kDa IN-RT intermediate

was made during maturation of the Gag1-Pol1 precursor in Ty1 VLPs and that Ty1 RT is active in the form of the Gag1-Pol1 precursor in yeast PR⁻ mutants [93]. More recently, Merkulov et al. [71] systematically mutagenized the cleavage sites between the protein domains of the Ty1 Gag1-Pol1 precursor to determine the role of individual protease cleavage sites in Ty1 retrotransposition. The ability of the IN*RT and PR*IN*RT fusion proteins to make amounts of Ty1 cDNA similar to wild-type level indicates that Ty1 RT retains its full activity in vivo when it is fused to the IN domain. There are precedents in retroviral and retrotransposon systems for RT-IN intermediates in the maturation of *pol* gene products and for the role of IN in RT activity. A role for the IN domain in RT activity has been demonstrated by the Sandmeyer's laboratory [81–82] for the Ty3 RT. C-terminal deletions and mutations in both N-terminal and C-terminal domains of Ty3 IN were found to severely affect the amount of reverse-transcribed Ty3 DNA associated with VLPs. This result, coupled with the observation that two forms of RT (the mature 55-kDa species and a 115-kDa RT-IN fusion protein) are detected by immunoblot analyses, led to the hypothesis that the Ty3 polymerase might be an RT/RT-IN heterodimer and implied a functional role of the interaction between IN and RT.

There are also examples in retroviral systems for heterodimeric RT/RT-IN proteins. Trentin et al. [80] have reported that the Human T-cell leukemia virus type 1 (HTLV-1) RT requires the presence of both RT (α subunit) and RT-IN (β subunit) for its activity. Since the HTLV-1 RT is not active in the absence of β, the RT-IN can be viewed as an integral part of RT. The active RT of the avian retrovirus RSV (Rous sarcoma virus) is also composed of α and β subunits [83]. These results suggest that interaction of the RT and IN domains is functionally significant and that the IN domain could play a role in the proper folding of RT. This could be achieved in cis when the IN domain is attached to the RT domain in the RT-IN fusion protein or in trans by interactions between the α and β subunits or by direct interaction between the individual mature IN and RT proteins. More structural studies of the retroviral RT-IN fusion protein and ongoing efforts to crystallize the Ty1 IN-RT enzyme will help to understand how the activities of retroviral and retrotransposon RTs are affected by the interactions between the RT and IN domains.

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