Expression of an active form of recombinant Ty1 reverse transcriptase in *Escherichia coli*: a fusion protein containing the C-terminal region of the Ty1 integrase linked to the reverse transcriptase–RNase H domain exhibits polymerase and RNase H activities

Marcelle WILHELM, Mansour BOUTABOUT and François-Xavier WILHELM[1]

Unité Propre de Recherche 9002 du Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex, France

Replication of the *Saccharomyces cerevisiae* Ty1 retrotransposon requires a reverse transcriptase capable of synthesizing Ty1 DNA. The first description of an active form of a recombinant Ty1 enzyme with polymerase and RNase H activities is reported here. The Ty1 enzyme was expressed as a hexahistidine-tagged fusion protein in *Escherichia coli* to facilitate purification of the recombinant protein by metal-chelate chromatography. Catalytic activity of the recombinant protein was detected only when amino acid residues encoded by the integrase gene were added to the N-terminus of the reverse transcriptase–RNase H domain. This suggests that the integrase domain could play a role in proper folding of reverse transcriptase. Several biochemical properties of the Ty1 enzyme were analysed, including the effect of $MgCl_2$, NaCl, temperature and of the chain terminator dideoxy GTP on its polymerase activity. RNase H activity was examined by monitoring the cleavage of a RNA–DNA template–primer. Our results suggest that the distance between the RNase H and polymerase active sites corresponds to the length of a 14-nucleotide RNA–DNA heteroduplex. The recombinant protein produced in *E. coli* should be useful for further biochemical and structural analyses and for a better understanding of the role of integrase in the activation of reverse transcriptase.

Key words: polymerization, retrotransposon, sequence comparison, template-primer, yeast.

INTRODUCTION

The genome of the yeast *Saccharomyces cerevisiae* contains five families of long-terminal-repeat (LTR) retrotransposons, Ty1–Ty5 [1]. They are structurally and functionally similar to eukaryotic retroviruses [2,3]. The Ty1 elements have a length of 5.9 kb, and consist of a large internal domain composed of two overlapping open reading frames, TyA and TyB, analogous to the retroviral *gag* and *pol* genes, flanked on either side by direct long terminal repeats. The life cycle of Ty elements begins with transcription of the integrated retrotransposon DNA. The Ty transcript is then packaged into the intracytoplasmic virus-like particles (VLPs) where it is converted into double-stranded DNA by the process of reverse transcription [4,5]. Both strands of the retrotransposon DNA are synthesized by the retrotransposon reverse transcriptase (RT) which is able to utilize both RNA and DNA as template and requires primers to initiate DNA synthesis. A specific host tRNA is used as a primer to initiate minus-strand cDNA synthesis and an RNase H (RH)-resistant oligoribonucleotide spanning a purine-rich sequence is used to prime plus-strand-DNA synthesis. RT activity has been demonstrated in VLPs purified from cells overproducing Ty1 elements. Attempts to obtain a soluble fraction of Ty1 RT from VLPs or to clone an active recombinant enzyme have been unsuccessful until now.

In the present paper we describe the expression, purification and initial biochemical characterization of an active recombinant Ty1 RT. DNA fragments encoding the RT open reading frame were synthesized by PCR and cloned into an *Escherichia coli* expression vector. We demonstrate that a fusion protein Ty1 integrase (IN)–RT–RH with a 115-amino-acid C-terminal fragment of the Ty1 integrase fused to the N-terminus of the RT–RH domain exhibits significant polymerase and RH activities. We have compared the biochemical features of the recombinant protein with the equivalent features of the Ty1 VLP-associated enzyme.

EXPERIMENTAL

Construction of *E. coli* plasmids expressing Ty1 proteins

The *E. coli* expression vector p6H (kindly given by S. Le Grice, NCI-Frederick Cancer Research and Development Center, Frederick, MD, U.S.A.) which encodes a hexahistidine array (His-tag) at the N-terminus of the recombinant proteins was used to clone the Ty1 RT [6]. The RT–RH domain spanning nts 3946–5562 of the Ty1-H3 coding sequence [7] and the three IN–RT–RH constructs spanning nts 3868–5562 (construct 1), nts 3601–5562 (construct 2) and nts 3511–5562 (construct 3) of the Ty1-H3 were amplified by PCR and cloned into the p6H vector.

The same 3′ amplification primer was used for all constructs. It contained a *Sal*I site and was complementary to the 3′ terminal coding sequence (including the stop codon) of the TyB open reading frame (ORF) (5′-ACGCGTCGACCTAATGAATCC-ATTTG-3′). Different 5′ amplification primers were used to amplify the RT–RH and IN–RT–RH domains. All 5′ primers contained a *Bam*HI site. They differed by the coding sequence for the N-terminal amino acids: 5′-CGCGGATCCGCTGTAAAA-

Abbreviations used: RT, reverse transcriptase; RH, RNase H; LTR, long terminal repeat; VLP, virus-like particle; IN, integrase; His-tag, hexahistidine; ORF, open reading frame; PR, protease; dd, dideoxy; HTLV-1, human T-cell leukaemia virus Type 1.

[1] To whom correspondence should be addressed (e-mail wilhelm@ibmc.u-strasbg.fr).

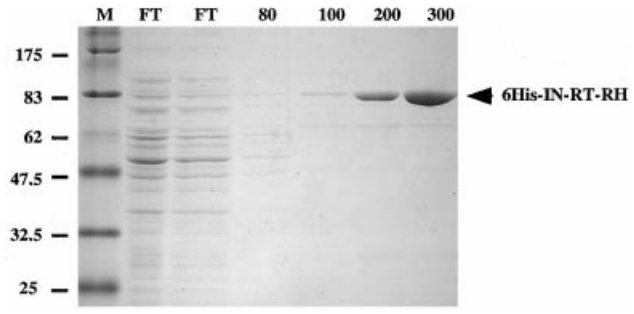

Figure 1 Purification of hexahistidine-tagged recombinant Ty1 IN-RT-RH (construct 2) by Ni^{2+}-nitrilotriacetic acid–agarose affinity chromatography

SDS/PAGE analysis of the proteins eluted from the affinity column by increasing concentrations of imidazole (80, 100, 200 and 300 mM) is shown. Lane M, molecular-mass markers (indicated in kDa on the left); lanes FT, flow-through fractions. The calculated molecular mass of the His_6–IN–RT–RH (construct 2) fusion protein is 76.4 kDa.

GCAGTAAAATC-3′ (RT–RH), 5′-CGCGGATCCTCACGA-GACACATGGAATACTAAG-3′ (IN–RT–RH construct 1), 5′-CGCGGATCCAATAATTCATCGCACAATATTG-3′ (IN–RT–RH construct 2) and 5′-CGCGGATCCAACAACAAAA-CTGTTCCGCAG-3′ (IN–RT–RH construct 3). The PCR amplification products were gel-purified, digested with *Bam*HI and *Sal*I and ligated into *Bam*HI–*Sal*I-digested p6H vector. Recombinant plasmids were introduced into component *E. coli* M15 cells.

Expression and purification of recombinant proteins

The Ty1 recombinant proteins containing a short array of histidine residues at their N-terminus were expressed in *E. coli* and purified by Ni^{2+} nitrilotriacetic acid–agarose (Qiagen) affinity chromatography as described by Le Grice et al. [6] (Figure 1). We did not expect that the His-tag would inhibit the activity of Ty1 RT, since, in all cases studied so far, it has been observed that inclusion of a polyhistidine extension in recombinant RTs has no deleterious effect on their polymerase or RH activities [6,8,9].

Purification of Ty1 VLPs

Ty1 VLPs were isolated from the yeast strain AGY9 (*MATa leu2Δ1 ura3-52 trp1Δ63 his4-539 lys2-801 spt3-202*) transformed with plasmid pJEF1105 [4,10]. The yeast strain and the plasmid were kindly provided by J. D. Boeke (Department of Molecular Biology and Genetics, Johns Hopkins University, Baltimore, MD, U.S.A.). Ty1 VLPs were purified by using the method described by Eichinger and Boeke [4]. RT activity was assayed in purified VLP preparations with added exogenous poly(rC)$_n$-oligo(dG)$_{12–18}$: 4 μl of purified VLPs were mixed with 16 μl of assay mix and incubated for 60 min at room temperature (22–24 °C). The standard assay mix (20 μl) contained final concentrations of 50 mM Tris/HCl, pH 7.8, 40 mM KCl, 20 mM $MgCl_2$, 0.05 % Nonidet P40, 0.15 μM dGTP, 8 mM 2-mercaptoethanol, 0.01 unit of poly(rC)$_n$–oligo(dG)$_{12–18}$ (where 1 unit of poly(rC)$_n$-oligo(dG)$_{12–18}$ is 50 μg) and 1 μCi of [α-^{32}P]dGTP. Incorporation of ^{32}P-radiolabelled dGTP into high-molecular-mass poly(dG) was determined by scintillation counting. Aliquots of the reaction mixture were spotted on to DE81 filters (Whatman). The filters were washed three times in 5 % Na_2HPO_4 to remove unincorporated [α-^{32}P]dGTP, washed once in deionized water and dried after one ethanol wash (75 % ethanol, 0.15 M NaCl).

RNA labelling, RNA template–DNA primer annealing and RH assay

A 311-nt single-stranded RNA template was prepared by T7 polymerase *in vitro* transcription of *Rsa*I-digested pJCB plasmid (kindly given by F. Brulé, Institut de Biologie Moleculaire et Cellulaire, Strasbourg, France). The transcript consists of 311 nt of the 5′ terminal region of HIV-1 RNA starting at the first nucleotide of the genomic RNA [11]. The RNA was radiolabelled by adding [α-^{32}P]UTP to the reaction mixture and purified by electrophoresis on a 8 % denaturing polyacrylamide gel. RNA was eluted from the excised gel slices with 0.5 M ammonium acetate/1 mM EDTA. The 35-nucleotide chemically synthesized RNA (kindly given by L. Jaeger, Institut de Biologie Moleculaire, Strasbourg, France) was labelled at the 5′ terminus with ^{32}P using [γ-^{32}P]ATP and T4 polynucleotide kinase. An alkaline ladder was prepared by treating the 5′-labelled RNA with sodium carbonate (0.1 M, pH 9) for 4 min at 90 °C.

To prepare the RNA template–DNA primer duplex used in RH assay, a 3 M excess of DNA primer was hybridized with the RNA template in a buffer containing 50 mM Tris/HCl, pH 7.8, 15 mM NaCl and 8 mM 2-mercaptoethanol. The mixture was heated at 90 °C for 1 min and then incubated for 5 min at 70 °C and 10 min at 42 °C. RH cleavage was initiated with $MgCl_2$ at a final concentration of 20 mM. After addition of the Ty1 or HIV-1 enzyme the samples were incubated for 10 min at 23 °C. The reaction was stopped by addition of formamide sample buffer. The products of the reaction were analysed on denaturing 8 % or 15 % (w/v) polyacrylamide gels.

RT assay of purified recombinant proteins

The purified Ty proteins were tested for RT activity by following the poly(rC)$_n$-oligo(dG)$_{12–18}$ directed incorporation of [α-^{32}P]dGTP. Unless otherwise stated, assay mix (20 μl) contained final concentrations of 50 mM Tris/HCl, pH 7.8, 15 mM NaCl, 20 mM $MgCl_2$, 0.15 μM dGTP, 8 mM 2-mercaptoethanol, 0.01 unit of poly(rC)$_n$-oligo(dG)$_{12–18}$ and 1 μCi of [α-^{32}P]dGTP. Incubation was for 30 min at 23 °C. Incorporation of ^{32}P-radiolabelled dGTP into high-molecular mass poly(dG) was determined as described above.

RESULTS AND DISCUSSION

Expression and purification of recombinant Ty1 RT

The enzymes essential for the replication and transposition of Ty1 elements are encoded by the TyB ORF (Figure 2). Coding domains for a protease (PR), an IN and RT–RH have been identified on the TyB gene. The TyB-encoded proteins are synthesized as part of a 190 kDa TYA–TYB precursor. The order of the protein domains on the TYA–TYB precursor is TYA–PR–IN–RT–RH. The TYA, PR, IN and RT–RH polypeptides are cleaved from the TYA-TYB polyprotein by the Ty1-encoded PR. The cleavage sites between TYA and PR, PR and IN, and IN and RT–RH have been characterized [12–14]. Moore and Garfinkel [15] have reported that the 5′ end of the RT–RH coding region starts at position 3946 of the transcriptionally active Ty1-H3 element [7]. The RT–RH coding region ends at the stop codon located at the 3′ end of the TyB ORF (nt 5562). Consequently we cloned the RT–RH coding region extending from nt 3946 to nt 5562 into the p6H *E. coli* expression vector. The recombinant RT–RH protein was

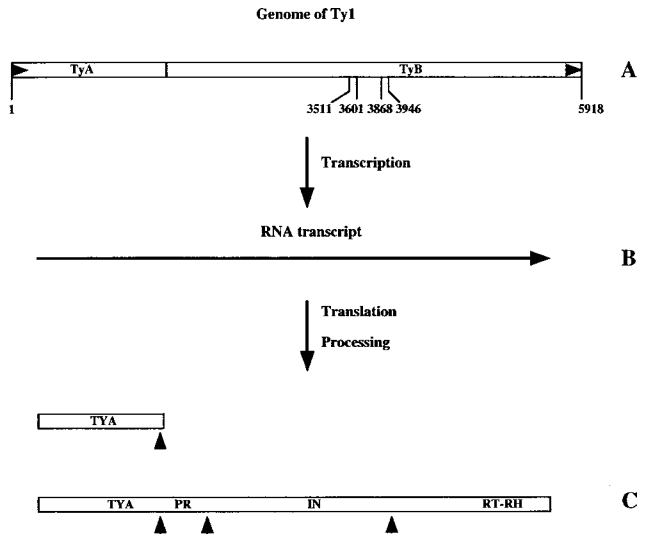

Figure 2 Map of Ty1 showing sites and co-ordinates relevant to the present study

(**A**) The Ty1 genome contains two LTRs (▶) flanking two partially overlapping open reading frames, TyA and TyB, which are analogous to the retroviral *gag* and *pol* genes. The transcriptionally active Ty1-H3 element is 5918 bp in length. The LTRs are 334 bp in length. The frameshift region separating TyA and TyB is indicated by a vertical line. The positions of the nucleotides at the 5′ end of the four constructs used in this study are indicated. (**B**) Transcription of the Ty1 element yields a major transcript of 5.7 kb. (**C**) The Ty1 RNA is translated to form two translation products, TYA and the TYA–TYB readthrough protein. The TYA–TYB protein contains TYA, a PR, an IN and a RT–RH domain. TYA and TYA–TYB are cleaved by the Ty1 PR to yield mature proteins. The cleavage sites are indicated by arrowheads (▲).

expressed, purified, and assayed for RT activity as described in the Experimental section. We found that the purified RT–RH protein encoded by nts 3946–5562 did not show any polymerase activity.

Several reports have implicated a role of the IN domain in the enzymic activity of some retroelement RTs [9,16,17]. We therefore constructed three IN–RT–RH fusion recombinant proteins containing fragments of the C-terminal region of the Ty1 IN attached to the N-terminus of the RT–RH domain: construct 1 had 26 residues from the IN C-terminal region fused to the RT–RH domain. One of the nuclear localization signals of Ty1 IN identified by Kenna et al. [18] and Moore et al. [19] was included in the N-terminal extension. Constructs 2 and 3 had 115 and 145 residues from the IN C-terminal region fused to the RT–RH domain respectively. The N-terminal region of constructs 2 and 3 ends at an NN motif, which could be a cleavage recognition site for the Ty1 PR. The three IN–RT–RH fusion proteins were expressed in *E. coli* and assayed for polymerase activity. Constructs 2 and 3 showed a high DNA polymerase activity, whereas construct 1 was inactive. In the experiments described below we used construct 2, which is the shortest protein with a high DNA polymerase activity, to characterize some biochemical features of the Ty1 RT.

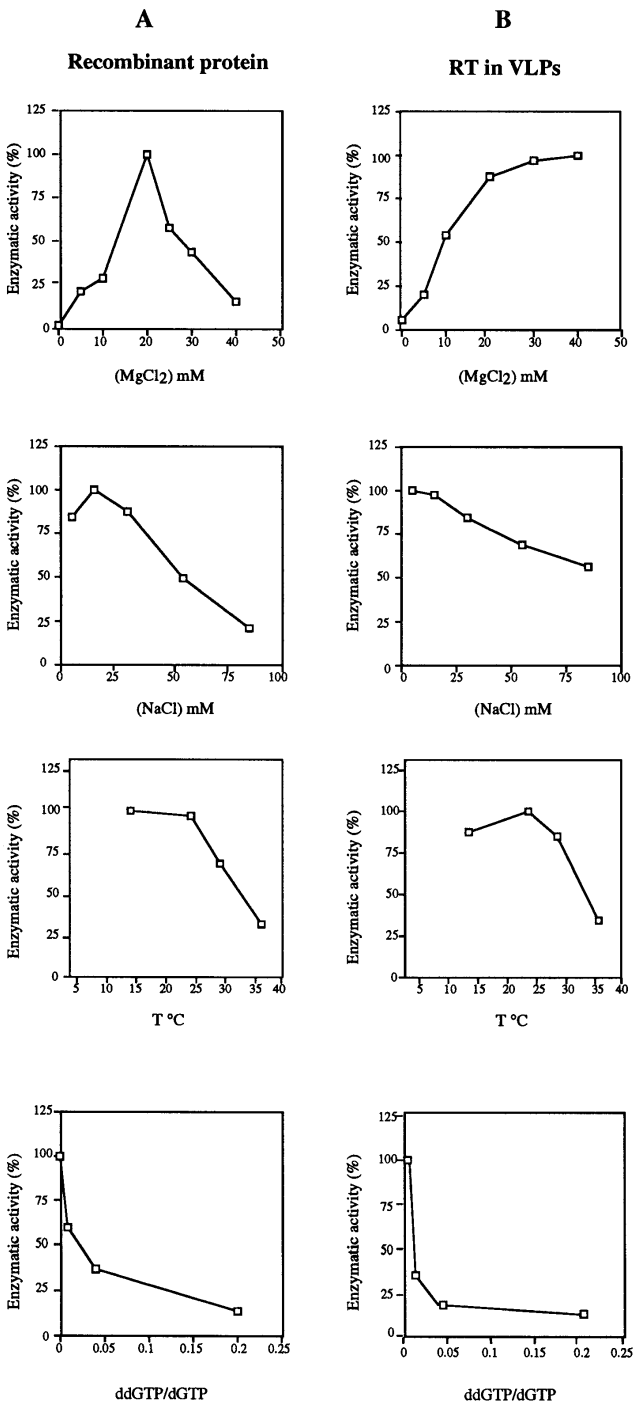

Figure 3 Effect of $MgCl_2$, NaCl, temperature and ddGTP on the polymerase activity of purified recombinant Ty1 IN–RT–RH protein and VLP-associated enzyme

Polymerase activity was measured as described in the Experimental section. For the recombinant protein, 100% activity is equal to 90 pmol of dGTP incorporated/μg in 30 min. For the VLPs, 100% activity is equal to 5 pmol of dGTP incorporated in 30 min for 4 μl of sucrose-gradient-purified VLPs.

Polymerase activity of the recombinant Ty1 IN–RT–RH enzyme

Four parameters of the RT polymerase activity were assessed using construct 2 of recombinant Ty1 IN–RT–RH and were compared with those of Ty1 VLP-associated enzyme. These parameters included the bivalent-cation requirement, the effects of NaCl and temperature on enzymic activity, and the sensitivity to inhibition by the chain terminator dideoxy GTP (ddGTP).

The Ty1 RT requires bivalent cations for optimal activity. The effect of Mg^{2+} and Mn^{2+} on the activity of Ty1 RT was examined.

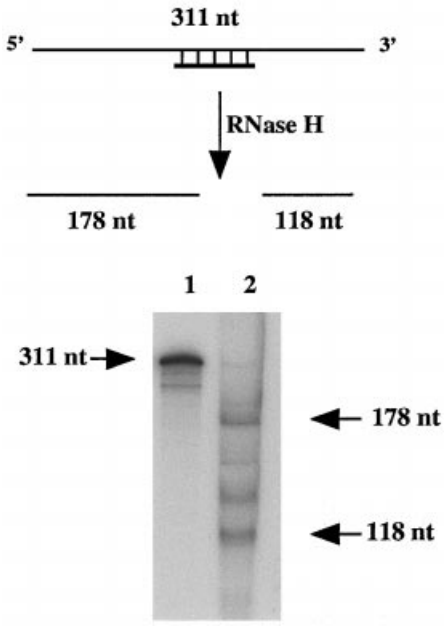

Figure 4 RH activity of the Ty1 IN–RT–RH protein

An internally ^{32}P-labelled 311 nt RNA template was hybridized with a molar excess of 18 nt DNA oligonucleotide primer and incubated with Ty1 enzyme as described in the Experimental section. The products of the reaction were analysed on a denaturing 8%-polyacrylamide gel. Polymerase-dependent cleavage by RH produced the expected 5′ fragment of 178 nt and 3′ fragment of 118 nt. Arrows indicate the positions of these fragments. A prominent polymerase-independent or non-specific cleavage product is also visible on the gel. A mock-treated RNA is shown in lane 1.

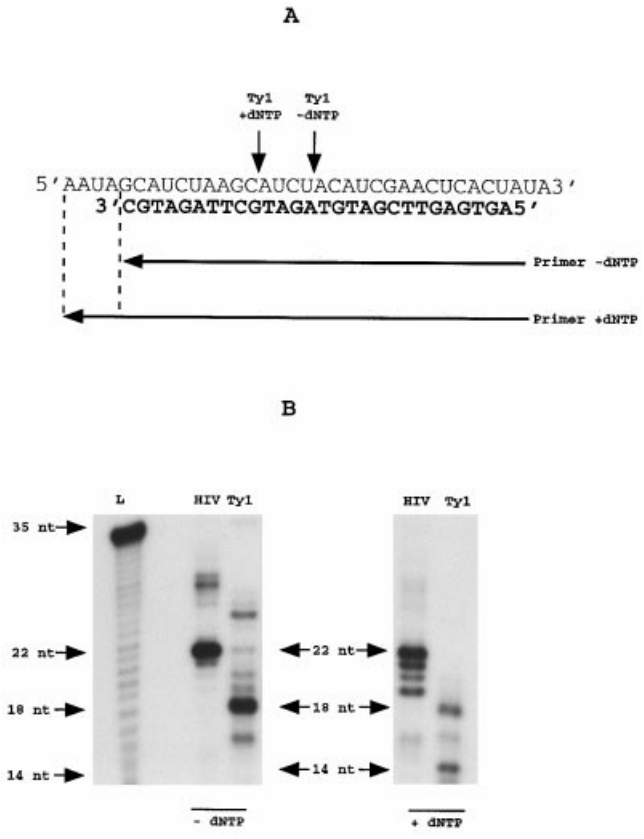

Figure 5 Interplay between the RH and polymerase active sites

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 (with or without dNTPs) to induce RH cleavage. In the presence of dATP and dTTP the primer was extended by 4 nt. The sites of cleavage by the Ty1 enzyme are indicated by arrows in (**A**). The cleavage products analysed on a denaturing 15%-polyacrylamide gel are shown in (**B**). Lane L, alkaline hydrolysis of the RNA template. HIV-1 or Ty1 enzymes were incubated with template–primer without any added dNTPs ('−dNTP'). The size of the main cleavage products are 22 nt and 18 nt for HIV-1 and Ty1 enzymes respectively. HIV-1 or Ty1 enzymes were incubated with template–primer, dATP and dTTP (+dNTP). The original 18 nt band and a 14 nt band are observed for the Ty1 enzyme. Cleavage bands at 22, 21, 20 and 19 nt are observed for the HIV-1 enzyme.

We found that the enzymic activity with Mg^{2+} was about threefold that with Mn^{2+} (results not shown). Therefore, in common with all other RTs studied, the Ty1 protein shows a preference for Mg^{2+} over Mn^{2+}. The Mg^{2+} response curve illustrated in Figure 3 demonstrates that the recombinant and VLP-associated enzymes have an absolute requirement for a bivalent cation. The recombinant enzyme exhibited a maximal activity at 20 mM Mg^{2+}, which decreased rapidly at higher Mg^{2+} concentrations. The activity of the VLP-associated enzyme was almost maximum at 20 mM Mg^{2+}, but did not decrease when the Mg^{2+} concentration was increased to 30 or 40 mM.

The effect of NaCl on the polymerase activity of recombinant and VLP-associated enzyme is shown in Figure 3. Both enzymes were inhibited by increasing concentrations of NaCl, but the VLP-associated enzyme was not inhibited to the same extent as the recombinant enzyme.

The polymerase activity of the recombinant enzyme was optimum at temperatures between 15 °C and 25 °C. The same temperature optimum was observed for the polymerase activity in VLPs (Figure 3). The low temperature optimum of the Ty1 enzyme can probably be correlated to the temperature-sensitivity of the Ty1 transposition process.

RTs are known to be sensitive to ddNTPs, which act as chain terminators. The effect of increasing concentrations of ddGTP on poly(rC)$_n$-oligo(dG)$_{12–18}$-directed polymerization of ^{32}P-labelled dGTP was tested. The titration curves shown in Figure 3 demonstrate that ddGTP is a strong inhibitor of Ty1 RT. In common with RTs from other eukaryotes, both recombinant and VLP-associated RTs were inhibited at low ddGTP concentration.

RH activity of the recombinant Ty1 IN–RT–RH enzyme

The RH activity of Ty1 enzyme was examined by monitoring the cleavage of an internally labelled 311 nt RNA transcript annealed to a complementary 18 nt oligodeoxyribonucleotide. The template–primer and the Ty1 enzyme were incubated to induce RH cleavage, as described in the Experimental section. Analysis of the RNA cleavage products on a denaturing gel showed that efficient cleavage was obtained with the recombinant Ty1 IN–RT–RH enzyme (Figure 4).

Next we analysed the interdependence between the RH and polymerase active sites of the Ty1 enzyme. To this end we used a 35 nt RNA template–28 nt DNA primer as substrate (Figure 5). The resulting complex contained a recessed 3′-terminus of DNA primer and four single-stranded RNA template bases. The 5′ end of the RNA was radiolabelled to allow one to reveal the RH cleavage products by gel electrophoresis. As shown in Figure 5(B) ('−dNTP'), RH cleavage of the Ty1 enzyme was specific for the RNA phosphodiester bond between nts 14 and 15 downstream from the nucleotide complementary to the 3′ ter-

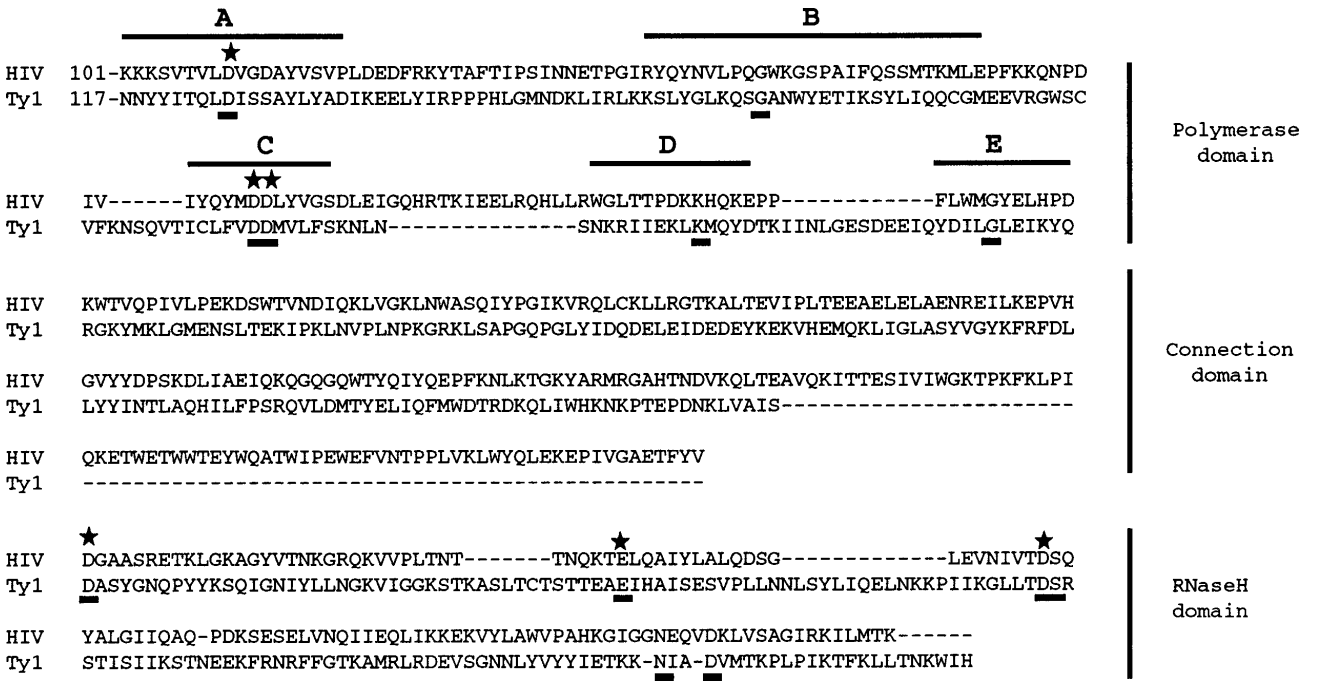

Figure 6 Sequence alignment of Ty1 and HIV-1 RTs

Three domains have been identified: the polymerase domain, the connection domain and the RH 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. [29]. The nearly invariant amino acid residues found in all RNA-dependent polymerases are underlined. The three catalytically essential D residues are highlighted by stars. The connection domain extends from the last residue in the conserved motif E to the first invariant residue of the RH domain [31]. The connection domains of HIV-1 and Ty1 contain 205 and 133 residues respectively. Alignment of the RH domains was as described by McClure [31]. The catalytically essential residues are indicated by stars. The nearly invariant amino acid residues found in all RH domains are underlined. The numbers at the beginning of the sequences indicate the number of residues from the N-terminus of the RT–RH omitted from the Figure.

minal nucleotide of the primer (the cleaved labelled RNA fragment has a length of 18 nt). In comparison, HIV-1 enzyme 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 RH cleavage and DNA polymerase active sites is 18–20 bp [20–27]. The effect of primer extension on RH cleavage was examined (Figure 5B, '+dNTP'). When dATP and dTTP were present in the reaction mixture, the primer was extended by four nucleotides. Interestingly, for the Ty1 enzyme, both the originally observed RH-cleavage band and a new band four nucleotides shorter were obtained. This new band results from RH cleavage of the RNA between nts 14 and 15 from the nucleotide complementary to the 3′ terminus of the extended primer. It is a polymerase-dependent cleavage site, as defined by Furfine and Reardon [28], since it is advanced upon primer extension and remains at a fixed distance from the extended primer terminus. In the case of HIV-1, the originally observed RH cleavage and three additional sites of RNA hydrolysis were observed when the primer was extended by four nucleotides. The specific cleavage site between nts 18 and 19 from the nucleotide complementary to the 3′ end of the elongated primer was not observed. This is in line with results of Furfine and Reardon [28], showing that the RH of HIV-1 does not always make all the cleavages upon primer extension.

Our results have shown that the distance between the RH and polymerase active sites of the Ty1 enzyme is 14 nucleotides. It is four nucleotides shorter than the distance measured for HIV-1 RT. We suggest that this difference can be explained by a difference of 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 RH domain by a connection subdomain which forms a groove between the two active sites and plays a role in binding of the primer–template [22]. Sequence comparison of Ty1 and HIV-1 RTs [29–31] shows that the connection domain of the Ty1 enzyme is shorter than the connection domain of the HIV-1 RT (Figure 6). This difference could explain the difference of length of primer–template which can be accommodated between the active sites of the two enzymes. Crystallization of Ty1 RT will allow this suggestion to be directly tested.

Concluding remarks

Replication of retrotransposons and retroviruses requires an RT capable of reverse-transcribing the RNA genomes into double-stranded DNA. Here we have reported on the expression and the purification of Ty1 RT with polymerase and RH activities. An active recombinant protein could be obtained only after adding amino acid residues encoded by the IN gene to the N-terminus of the RT–RH domain. This suggests that the interaction between the C-terminal region of the IN and the RT–RH domain is important for the function of RT. It has been previously reported that a 140 kDa IN–RT–RH intermediate was observed during maturation of the TYA–TYB precursor. Thus it is possible that an interaction between the RT–RH and IN is also necessary for the activity of RT in VLPs. A mature 60 kDa protein encoded by the RT–RH ORF co-fractionates with Ty1 VLPs. The possibility that an interaction in *trans* between this protein and IN is necessary for its activity cannot be excluded. Interestingly a role for the IN domain in RT activity has been demonstrated by

Kirchner and Sandmeyer [16] for *S. cerevisiae* Ty3 retrotransposon; it is noteworthy that a decreased RT activity of Ty3 mutant VLPs relative to wild-type was observed for IN C-terminal deletion mutants, despite a different TyB gene organization (in Ty3 the order of RT–RH and IN domains is RT–RH–IN, whereas it is IN–RT–RH in Ty1). There are precedents in retroviral systems for RT–RH–IN intermediates in the maturation of *pol* gene products and for the role of integrase in RT activity. For example, Trentin et al. [9] have reported that the Human-T-cell-leukaemia-virus-Type 1 (HTLV-1) RT requires the presence of both RT–RH (α-subunit) and RT–RH–IN (β-subunit) for its activity. Since the HTLV-1 RT is not active in the absence of the β subunit, the RT–RH–IN can be viewed as an integrant part of RT transcriptase. The active reverse transcriptase of Rous sarcoma virus, an avian retrovirus, is also composed of α- and β-subunits [17]. These results suggest that interaction of the RT–RH 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–RH domain or in *trans* as part of heterodimeric RTs containing α- and β-subunits or by direct interaction between the mature IN and RT–RH proteins. Ongoing efforts to crystallize the Ty1 RT, in addition to biochemical and genetic analyses, will help us to understand how the polymerase domain of Ty1 RT is affected by its interaction with the IN domain.

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