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Catalytic Features of the Recombinant Reverse Transcriptase of Bovine Leukemia Virus Expressed in Bacteria

Michal Perach and Amnon Hizi[1]

Department of Cell Biology and Histology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel

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We have expressed the recombinant reverse transcriptase (RT) of bovine leukemia virus (BLV) in bacteria. The gene encoding the RT was designed to start at its 5′ end next to the last codon of the mature viral protease, namely the amino terminus of the RT matches the last 26 codons of the *pro* gene and is coded for by the *pro* reading frame. The RT sequence extends into the *pol* gene, utilizing the *pol* reading frame after overcoming the stop codon by adding an extra nucleotide (thus imitating the naturally occurring frameshift event). Hence we have generated a transframe polypeptide that is a 584-residues-long protein (see Rice, Stephens, Burny, and Gilden (1985) *Virology* 142, 357–377). This protein was partially purified after adding a six-histidine tag and studied biochemically testing a variety of parameters. The enzyme exhibits all activities typical of RTs, i.e., both RNA- and DNA-dependent DNA polymerase as well as a ribonuclease H (RNase H) activity. Unlike most RTs, the BLV RT is enzymatically active as a monomer even after binding a DNA substrate. The enzyme shows a preference for Mg^{2+} over Mn^{2+} in both its DNA polymerase and RNase H activities. BLV RT is relatively resistant to nucleoside triphosphate analogues, which are known to be potent inhibitors of other RTs such as that of HIV.

INTRODUCTION

Bovine leukemia virus (BLV) is a naturally occurring exogenous B-cell lymphotropic retrovirus. This infectious virus is the etiological agent of enzootic cattle leukosis, characterized by an initial persistent lymphocytosis, which is followed by the occurrence of clonal lymphoid B-cell tumors after a long latency (Ghysdael *et al.,* 1985). BLV can infect *in vitro* a variety of cells and can propagate in different animal species. It is distantly related to the human lymphotrophic viruses type I and type II (HTLV-1 and HTLV-2, respectively), forming a subfamily of transactivating retroviruses (Coffin, 1996). These complex retroviruses contain at their 3′ end of genome the regulatory genes *tax* and *rex*. The presence of both Rex and Tax proteins are required for viral replication (Green and Chen, 1994; Kettmann *et al.,* 1994). These viruses also show nucleotide sequence similarities. Nevertheless, BLV and HTLVs do not infect the same cell types because they probably bind different cell receptors.

The process of reverse transcription is the major early intracellular event that is critical to the life cycle of all retroviruses. This stage in the synthesis of the proviral double-stranded DNA is catalyzed by a single viral enzyme, the reverse transcriptase (RT). The plus-strand viral RNA is copied by the RNA-dependent DNA polymerase (RDDP) activity of RT, producing RNA • DNA hybrids. Concurrently with this DNA synthesis, the intrinsic RNase H activity of RT specifically hydrolyzes the RNA in these heteroduplexes. Finally, the plus-strand DNA is synthesized by copying the first DNA strand by the DNA-dependent DNA polymerase (DDDP) activity of RT (with fragments of the original viral RNA serving as primers for DNA synthesis of the second DNA strand) (Skalka and Goff, 1993; Coffin *et al.,* 1997). The pivotal role of RT in the life cycle of retroviruses has made it a major target for the development of RT inhibitors as antiretroviral drugs (De Clercq, 1995; Coffin *et al.,* 1997). The molecular, structural, and catalytic properties of retroviral RTs have been the focus of numerous recent studies, including three-dimensional crystal studies (e.g., Kohlstaedt *et al.,* 1992; Jacobo Molina *et al.,* 1993; Georgiadis *et al.,* 1995; Huang *et al.,* 1998). A major effort was devoted to the research of the RTs of the human immunodeficiency viruses type 1 and type 2 (HIV-1 and HIV-2, respectively), the viruses responsible for acquired immunodeficiency syndrome (AIDS) because most anti-AIDS drugs approved so far for the treatment of this disease are inhibitors of the viral RT. Because of the rapid emergence of drug-resistant HIV RT variants, the development of novel potent and specific inhibitors of HIV RT is still a chief goal in the chemotherapy of AIDS. Targeted drug designs depend on a better understanding of the general structure and function of retroviral RTs. Therefore the investigation of RTs of other retroviruses is very likely to expand our understanding of the catalytic properties of these closely related proteins.

Almost all research on the structure and enzymatic features of RTs was done on the recombinant proteins

[1] To whom reprint requests should be addressed. Fax: 972–3-6407432. E-mail: ahizy@ccsg.tau.ac.il.

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expressed in large amounts in bacteria. BLV RT has not been expressed as a recombinant enzymatically active protein, and there are only very few reports with a partial characterization of the RT activity in virions of BLV (Gilden *et al.,* 1975; Mamoun *et al.,* 1981; Demirhan *et al.,* 1996). Therefore it was of interest to express recombinant BLV RT and to study its biochemical properties. We report here on such a study. When the outlined research of BLV RT was already underway, two very recent reports were published on the recombinant expression of the RT HTLV-1, which is closely related to BLV (Owen *et al.,* 1998; Trentin *et al.,* 1998). This allowed us to compare the strategies of cloning the RT-expressing genes and the basic features of the various RTs. The comparison, discussed below, reveals basic differences between the recombinant BLV RT and either one of the two recombinant RTs of HTLV-1 as well as between the two HTLV-1 RT species.

RESULTS

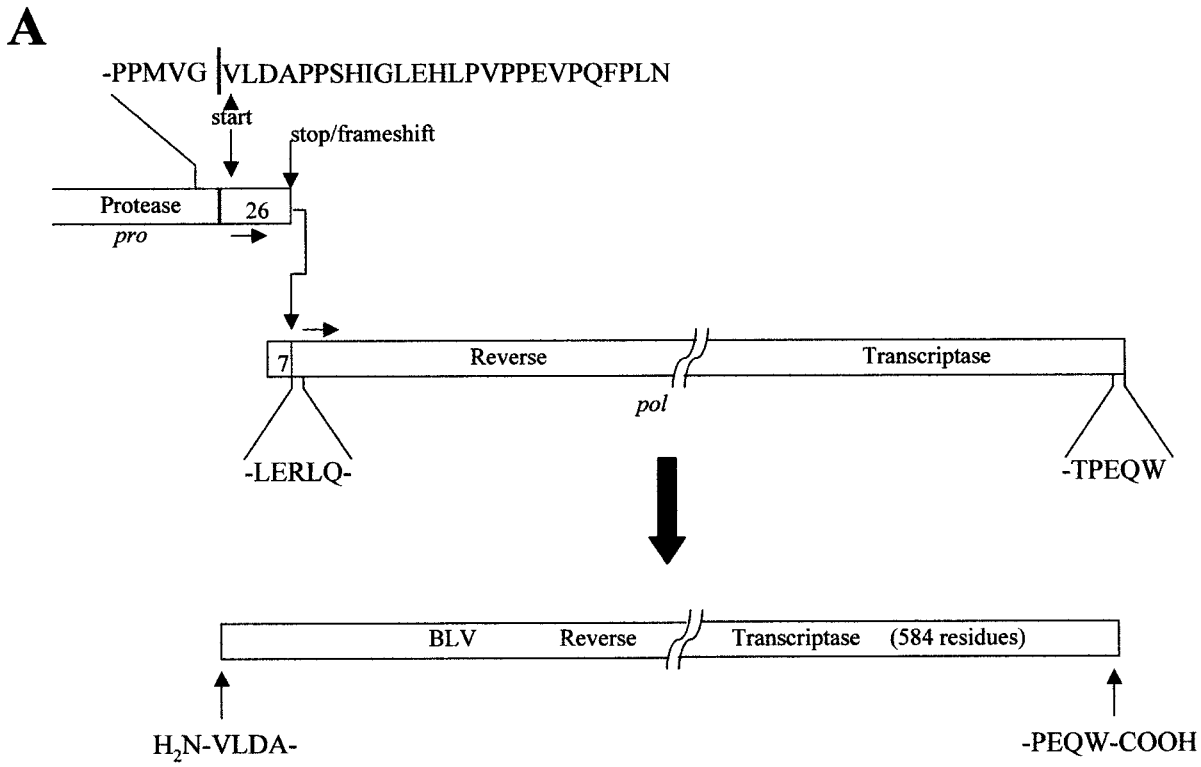
Expression of enzymatically active BLV RT

Rationale. The translation of retroviral reverse transcriptases is a relatively complex process because in most retroviruses it involves either one or two frameshifting events. The first reading frame of the genomic RNA, which serves as a messenger RNA, encodes for the polyprotein precursor of the Gag proteins. A Gag-Protease polyprotein precursor is synthesized as a result of overcoming the stop codon at the end of the *gag* gene by a frameshift event at the −1 direction. In BLV, a second frameshifting event is necessary to allow the synthesis of the Gag-Pro-Pol polyprotein precursor, which serves also as a precursor for the Pol-related enzymes, the RT and integrase (IN) (Jacks, 1990; Coffin *et al.,* 1997). The actual amino acid sequences at the carboxyl and amino termini of the virus-purified RTs have served as a lead for designing the gene coding for recombinant RTs (e.g., Hizi and Hughes, 1988; Hizi *et al.,* 1988). In the absence of such sequence information, sequence homologies to other known RTs were also used as a guide for the design of RT-coding genes (e.g., Hizi *et al.,* 1991a; Shaharabany *et al.,* 1993). The carboxyl terminus of the protease of all retroviruses studied so far was found to be next to the amino terminus of the RT. Hence another approach, employed in the strategy of planning the BLV RT-encoding gene in the present study, was to deduce the amino terminus of the protein from the carboxyl terminus of the protease, assuming that the two are adjacent as in other retroviruses.

The sequence analysis of the virus-purified BLV protease and its comparison with the nucleotide sequence of BLV genome (Rice *et al.,* 1985; Yoshinaka *et al.,* 1986) indicates that the codon for the last carboxyl terminal residue of the mature viral protease is located at the 26th codon upstream to the 3′ end of the *pro* open reading frame. Therefore based on the assumption that the amino terminus of the RT is adjacent to the carboxyl terminus of the protease, the amino terminus of the RT is predicted by us to be H_2N-Val-Leu-Asp-Ala-Pro- encoded by nucleic acid sequence starting in the *pro* gene at position 2055 in the published BLV sequence (Rice *et al.,* 1985). Consequently, BLV RT is expected to be a transframe protein. A similar approach was employed recently in designing the amino terminus of recombinant HTLV-1 RT (Trentin *et al.,* 1998). The frameshift site in BLV RT was predicted to be after codon 26 at −1 direction based on the consensus for retroviral frameshift sites (Hatfield *et al.,* 1992), allowing the translation of the rest of the protein from the *pol* open reading frame. The carboxyl terminus of the BLV RT was predicted based on both homology to termini of other RTs as well as on the putative protease cleavage site. This sequence is -Thr-Pro-Glu-Gln-Trp-COOH (corresponding to the sequence that ends in nucleotide 3805 in the BLV sequence). In all, the recombinant RT is expected to be 584-amino-acid residues long (Fig. 1A). To substantiate the selection of the right carboxyl terminus, we have also expressed an RT–IN fused protein, which ends at the natural integrase stop codon (starting at position 4667) and has a total length of 871 residues. This recombinant protein showed a DNA polymerase activity comparable to that of the 584-long RT (data not shown). Therefore we have assumed that there are no significant truncations at the carboxyl terminus, and hence our initial prediction of the sequence of this terminus is quite right.

Vector constructions. The overall strategy of the vector construction is described schematically in Fig. 1B. All DNA fragments were synthesized *in vitro* by PCR as described under Materials and Methods. The frameshifting event required for the expression of BLV RT does not normally occur in bacteria and certainly not in the position appropriate for the synthesis of authentic RT in the infected mammalian cells. Therefore as performed recently by us for the expression of MMTV RT (Taube *et al.,* 1998), we have mutated the RT-encoding gene. This was done by adding an extra cytosine downstream to the sequence TTTAAAC, which codes for the RNA translational frameshift site (between nucleotides 2132 and 2133 in the BLV genome). This procedure necessitated the initial PCR amplification of the RT-coding gene as two separate DNA fragments with some overlapping in one of which an additional dC was introduced (Fig. 1B).

The first DNA fragment (corresponding to nucleotides 2055–2465 in the BLV genome) was PCR-amplified using a 5′ end primer with an *Nco*I site, which introduces an initiation methionine adjacent to the sequence coding for the amino terminus of BLV RT (H_2N-Val-Leu-Asp-Ala-Pro-) (PCR1). The 3′ end primer matched the sequences of the BLV RT genome followed by an *Hin*dIII restriction site. The DNA fragment was cleaved with *Nco*I and *Hin*dIII



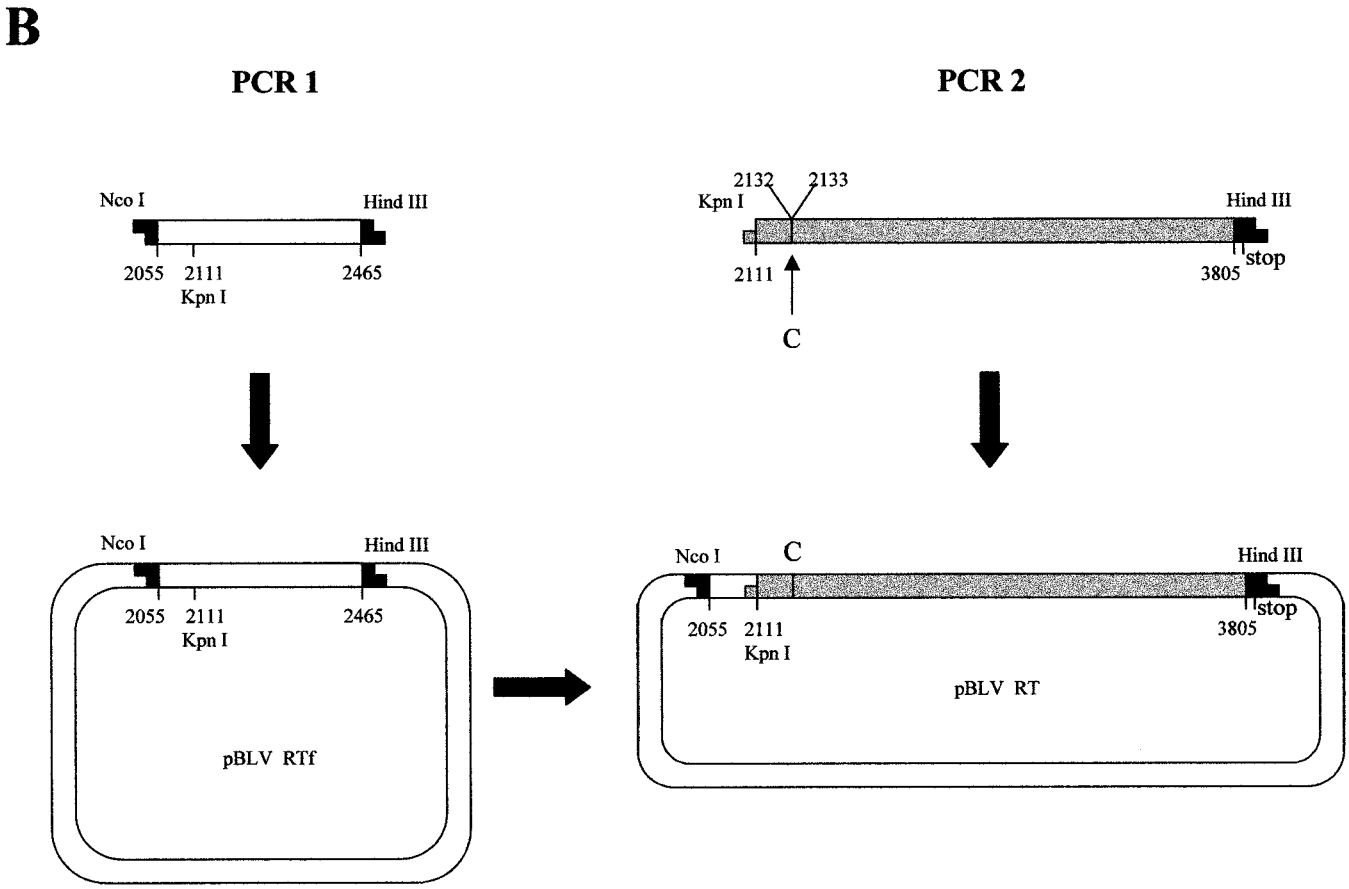


FIG. 1. Schematic description of the strategy and method of cloning the BLV RT-encoding gene into the pUC12N expression vector. (A) Presentation of the retroviral gene coding for BLV RT. The length of the polypeptide fragments are marked by the numbers of the residues. We have used the single letter codes for the amino acid sequences. (B) The method of constructing the BLV RT-expressing vector. The methodology is detailed under "Results". The blackened boxes represent sequences not present in the original BLV genome which were added by the PCR. The numbering of all nucleotide positions are those of the BLV genome according to Rice *et al.* (1995).

and subcloned into the pUC12N plasmid cleaved by the same restriction enzymes, generating a plasmid entitled pBLV RTf (see Materials and Methods). The second DNA fragment was PCR-synthesized using a 5′ end primer that introduced an extra C between nucleotides 2132 and 2133 (PCR 2). The 3′ end primer matched the BLV genome sequence up to nucleotide 3805, followed by a translation termination codon and an *Hin*dIII restriction site. The DNA fragment (~1750 bp long), which overlaps on its 5′ end the 3′ end of the first PCR-synthesized BLV RT DNA fragment, was cut by *Kpn*I and *Hin*dIII. The overlapping sequence contains a *Kpn*I site at the sequence corresponding to position 2111. Therefore the cleaved fragment was introduced into the *Kpn*I- and *Hin*dIII-cleaved plasmid harboring the first DNA segment (pBLV RTf), generating the final expression vector, pBLV RT (see Fig. 1B).

The DH5α bacteria transformed with the right plasmid were tested for the overexpression of BLV RT by checking the presence of the recombinant protein in whole cell lysates using SDS–PAGE analysis and by monitoring the poly(rC)$_n$ · oligo(dG)$_{12-18}$ -directed polymerase activity in bacterial extracts (see Materials and Methods). The *Nco*I–*Hin*dIII 1758-bp DNA inserts from positive clones were subcloned into pUC112N6H plasmid, the expression vector with the six-histidine tag sequence (see Materials and Methods).

Purification of the recombinant BLV RT

The bacterial clones harboring the pUC112N6H RT-expressing plasmid (see above) were examined for the level of RT activity and analyzed by SDS–PAGE. There was a polypeptide protein band with an apparent molecular weight of ~65 kDa that was not present in control *Escherichia coli* with a plasmid without the BLV RT gene insert (not shown). This molecular weight is compatible with a recombinant protein, which is 584 residues (plus six histidines and one methionine) long. The protein was purified by a fast purification method taking advantage of the presence of the six-histidine tag. Previous experiences with a variety of retroviral RTs have shown that recombinant RTs, tagged with six-histidine residues at either amino or carboxyl termini, are as active as the counterpart RT molecules with no histidine tags (unpublished results). Therefore there was no need to remove these extra sequences from the purified BLV RT. The purification procedure, employing Ni–NTA agarose affinity chromatography followed by a CM–Sepharose ion exchange chromatography, was described in detail under Materials and Methods. The typical quantitative purification is summarized in Table 1.

The first purification step resulted in a substantial increase (183-fold) in the specific activity of RT, accompanied by a 2.7-fold increase in the total activity relative to that of the crude lysate. The second purification step resulted in an additional considerable increase in the specific activity (of ~90-fold). Altogether, there is an overall 73-fold increase in the total DNA polymerase activity throughout the whole purification process, strongly suggesting the removal of potent RT inhibitors. Similarly, in the purification process of recombinant MMTV RT, both column chromatography steps led to increases in the total RT activity, suggesting also removal of inhibitors in both steps (Taube *et al.,* 1998). Unfortunately, unlike previous purifications of recombinant RTs with similar columns (including that of MMTV RT), an SDS–PAGE analysis of BLV RT revealed significant amounts of contaminating proteins (data not shown). Hence we conclude that despite the high increase in the specific activity of the RT (of ~16,380-fold), the final preparation of the BLV RT used was only partially pure. All contaminating protein polypeptides were bound to the Ni–NTA column, although proteins from lysates of control bacteria (prepared by the same extraction method) were shown not to bind to such columns (data not shown). Therefore we assume that the contaminating proteins are either of bacterial origin and were bound to the column *via* the histidine-tagged BLV RT molecules (by interacting with them noncovalently) and/or that proteolytically cleaved BLV RT-derived polypeptides bind the column *via* the retained histidine-tagged amino terminus. A partial support for the latter alternative might be the fact that most contaminating polypeptides were with apparent molecular weights smaller than the 65-kDa full-length BLV RT polypeptides and that other recombinant RTs were shown to be quite sensitive to bacterial proteases. This issue of the source of the contaminating

TABLE 1

Quantitative Summary of the Purification of Recombinant BLV RT

Purification step	Total protein (mg)	Total DNA polymerase activity (units)	Specific DNA polymerase activity (units/mg protein)
Crude bacterial extract	152	3.2×10^3	2.1×10^1 (1)
Ni–NTA agarose column eluate	2.24	8.6×10^3	3.84×10^3 (183)
CM–Sepharose column eluate	0.68	234×10^3	344×10^3 (16,380)

Note. The DNA polymerase activity is expressed in pmoles of [^{3}H]dGTP incorporated into the TCA-insoluble material in 30 at 37°C in the poly(rC)$_n$ · oligo(dG)$_{12-18}$-directed reactions as described under Materials and Methods. The different purification steps are described also under Materials and Methods. Protein concentrations were determined using bovine serum albumin as a protein standard (Bradford, 1976). The figures in parenthesis are the fold of increase in the specific activity of the purified protein relative to that of the crude bacterial lysate.

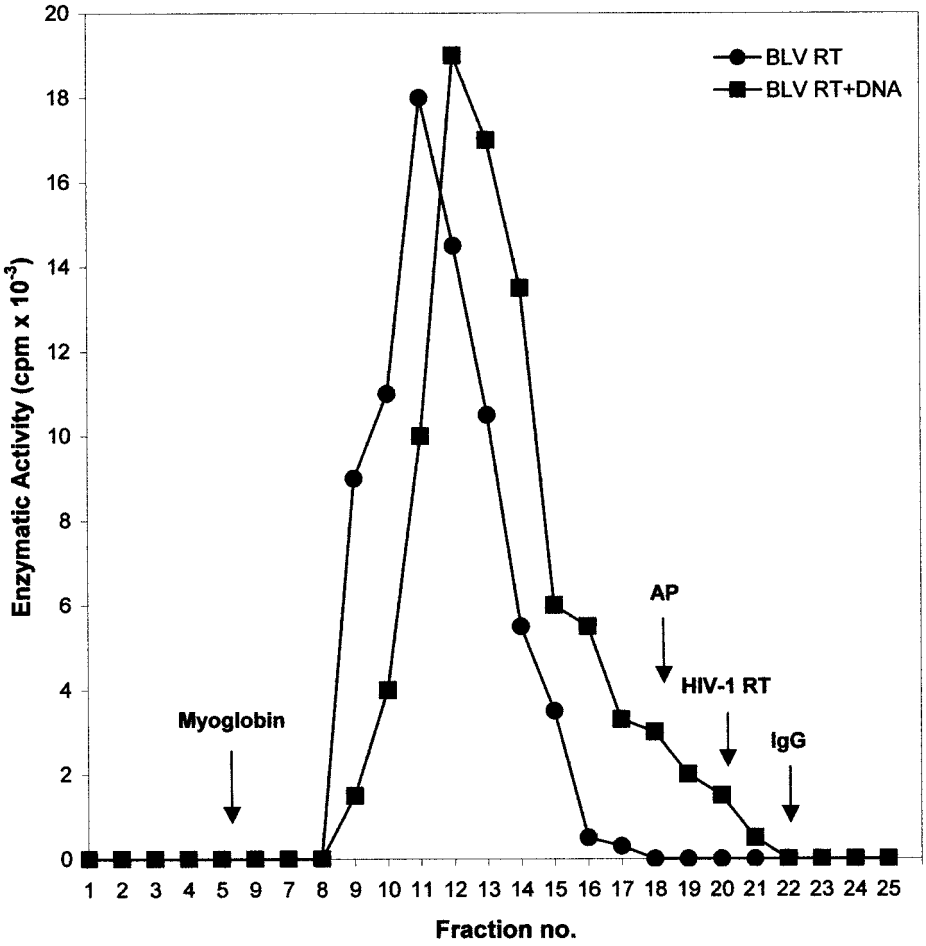


FIG. 2. Determination of the molecular size of the enzymatically active BLV RT by ultracentrifugation through glycerol gradients. Linear gradients of 20–35% (v/v) glycerol were prepared in 30 mM Tris–HCl, 0.1 M NaCl, 0.2% Triton X-100, 20 μg/ml BSA, and 5 mM DTT, centrifuged, and analyzed as described in detail previously (Hizi and Joklik, 1977; Taube *et al.,* 1998). (●) Free BLV RT. (■) BLV RT bound to double-stranded 48mer synthetic oligonucleotides. The preparation sequences and preincubation conditions of the BLV RT with these synthetic DNA were also described by us previously (see Taube *et al.,* 1998, Fig. 3). Molecular size markers were analyzed in parallel centrifuge tubes. These markers were: myoglobin, molecular mass 16.9 kDa (measured by $A410_{nm}$); bacterial alkaline phosphatase (AP), molecular mass 86 kDa (assayed using *p*-nitrophenyl phosphate as a substrate at $A405_{nm}$) HIV-1 RT, molecular mass 110 kDa (assayed by the routine BLV RT RDDP assay), and immunoglobulin G(IgG), molecular mass ~150 kDa. The positions of the peaks of these marker proteins are indicated by arrows.

proteins, their possible association with BLV RT, and methods to remove them will be studied in the future.

Subunit composition of the catalytically active BLV-RT

Most RTs studied so far are heterodimers, where the smaller subunits are derived from the larger ones by a cleavage toward their carboxyl termini by the retroviral protease (Skalka and Goff, 1993; Coffin *et al.,* 1997). One exception is the RT of murine leukemia virus (MLV), which is composed of monomers of ~71 kDa, although it was suggested that the enzyme dimerizes after binding the DNA substrate (Telesnitsky and Goff,1993). A second exception is the recently studied MMTV RT. Here, the RT is a 66-kDa monomer even after binding to its cognate DNA substrate (Taube *et al.,* 1998).

The purified BLV RT was analyzed from the molecular mass of the enzymatically active protein, employing ultracentrifugation through glycerol gradients under non-denaturating conditions along with known protein markers with known molecular masses (Fig. 2). We have confirmed that the gradient conditions preserved the authentic subunit pattern of RTs by testing, in addition to BLV RT, the sedimentation pattern of heterodimeric p66/p51 HIV-1 RT (molecular mass of ~110 kDa). As shown in Fig. 2, the DNA polymerase activity of BLV RT sediments as a single peak located between the myoglobin (M.W. 16.9 kDa) and alkaline phosphatase (AP) (M.W. 86 kDa) markers. The sedimentation of HIV-1 RT is substantially faster, between the alkaline phosphatase and the immunoglobulin G (IgG) (M.W. 150 kDa) markers. This strongly suggests that BLV RT is a monomeric enzyme in solution under conditions where HIV-1 RT is a dimer. Because it

was reported that monomeric MLV RT dimerizes in solution after binding to the DNA substrate, we have also examined whether the recombinant BLV RT shows a similar feature. Consequently, we have incubated the BLV RT with a molar excess of double-stranded oligomeric DNA prior to the sedimentation analysis. The RT bound to DNA also sediments as a monomer. It should be noted that the RT–DNA complex sediments faster than free monomeric RT, a phenomenon that can be explained by the additional mass of the DNA in the RT–DNA complex (48 bp long) and/or by a difference in the folding of the RT after binding the DNA. This supports the notion that all the RT molecules were bound to the DNA substrate. The behavior of BLV RT shown in Fig. 2 is practically indistinguishable from the behavior of monomeric MMTV RT because the complex of monomeric MMTV RT–DNA sedimented faster than the free monomeric RT (see Fig. 3 in Taube *et al.,* 1998).

The relative DNA polymerase and RNase H activities of BLV RT

All RTs studied so far are multifunctional, possessing RDDP, DDDP, and RNase H activities (Hizi *et al.,* 1977, 1991b; Hizi and Yaniv, 1980; Skalka and Goff, 1993; Rubinek *et al.,* 1994; Taube *et al.,* 1998). We have tested the purified BLV RT for all activities using a variety of polynucleotide substrates commonly suitable for assaying RT activities. Furthermore the divalent cation (Mg^{+2} *vs* Mn^{+2}) preference was checked for each substrate (Table 2). As for the DNA synthesis, of all substrates tested poly(rA)$_n$ · oligo(dT)$_{12-18}$ is the most efficient one. This finding is compatible with the substrate preference of most RTs studied (Skalka and Goff, 1993). Similar to other RTs, the substrate that is considered the most specific for RT-directed DNA polymerase, poly(2′-*O*-methylC)$_n$ · oligo(dG)$_{12-18}$, is the least efficient relative to all other substrates studied. Of substrates employed for assaying the DDDP activity, poly(dC)$_n$ · oligo(dG)$_{12-18}$ is shown to be the most efficient substrate, even higher than the RNA · DNA counterpart substrate, poly(rC)$_n$ · oligo(dG)$_{12-18}$. The recombinant BLV RT has also a significant RNase H activity with the synthetic substrate [^{3}H]poly(rA)$_n$ · poly(dT)$_n$, which is the most commonly used for assaying this activity.

Most DNA polymerases including all RTs are strictly dependent on the presence of divalent cations. Most RTs prefer Mg^{+2} over Mn^{+2} with the most outstanding exception are the RTs of mammalian type C retroviruses, such as MLV RT, which prefers Mn^{+2}. The BLV RT displays for all its catalytic activities a marked preference for Mg^{+2} over Mn^{+2} (Table 2). The ratio between the Mg^{+2}- and Mn^{+2}-dependent activities varies with the substrate and activity assayed. Thus the highest ratio is observed in the RDDP assay with poly(rA)$_n$ · oligo(dT)$_{12-18}$ (~12-fold) and the lowest one is detected in the RNase H activity (of ~12-fold).

TABLE 2

Catalytic Activities, Substrate Specificities, and Divalent Cation Preference of BLV RT

Activity	Substrate	Specific activities (pmole per mg protein) Mg^{+2}	Mn^{+2}
RDDP	Poly(rA)$_n$ · oligo(T)$_{12-18}$	1077.4	90.7
	Poly(rC)$_n$ · oligo(dG)$_{12-18}$	397.3	77.6
	Poly(2′-O-methylC)$_n$ · oligo(dG)$_{12-18}$	59.1	21.1
DDDP	Poly(dA)$_n$ · oligo(dT)$_{12-18}$	29.1	8.5
	Poly(dC)$_n$ · oligo(dG)$_{12-18}$	528.2	74.8
	Activated DNA	71.2	19.2
RNase H	[^{3}H]poly(rA)$_n$ · poly(dT)$_n$	7.1	2.1

Note. The specific activities are expressed either in pmoles dNTP incorporated into the nascent DNA strands (for DNA synthesis, namely RDDP and DDDP) or in pmoles [^{3}H]dAMP released from the substrate (in the case of RNase H), in 30 min at 37°C. Activated DNA was prepared as described under Materials and Methods. In the assay with this template, the dNTP incorporation was calculated for all dNTPs incorporated, assuming equimolar incorporation. All synthetic template · primers were at final concentrations of 5 μg/ml. The reactions were conducted in the presence of either 8 mM $MgCl_2$ or 1 mM $MnCl_2$. All values presented are averages of at least two independent experiments, after subtracting the background levels with no RT present in the assay tubes. The standard deviations for all results were usually 10% of the average values presented.

pH optimum for the catalytic activities of BLV RT

We have determined the dependence of the BLV RT catalytic activities on the pH of the reaction mixtures (Fig. 3). The DDDP exhibited a relatively wide range of pH dependence peaking at pH 7.0–8.0. The RDDP activity is more sensitive to lowering the pH than the DDDP. Both RDDP and DDDP functions are relatively insensitive to high pH values; thus only 40% of the activity was lost at pH 10. The pH-dependence curve for the RNase H activity is more symmetric with a sharper peak at around pH 8.0. At both pH 7.0 and 9.0, the activity drops to ~60% of the highest one at pH 8.0.

Kinetic constants of the DNA polymerase activities of BLV RT

To study the steady-state kinetic parameters of the recombinant BLV RT, we have calculated the K_m values for both RDDP and DDDP activities. These constants were derived from the double reciprocal curves of the initial velocities as a function of substrate concentrations (Table 3). The K_m values for the RDDP activity were calculated for dGTP and dTTP [in the poly(rC)$_n$ · oligo(dG)$_{12-18}$- and poly(rA)$_n$ · oligo(dT)$_{12-18}$-directed DNA synthesis, respectively] and for the two synthetic prim-

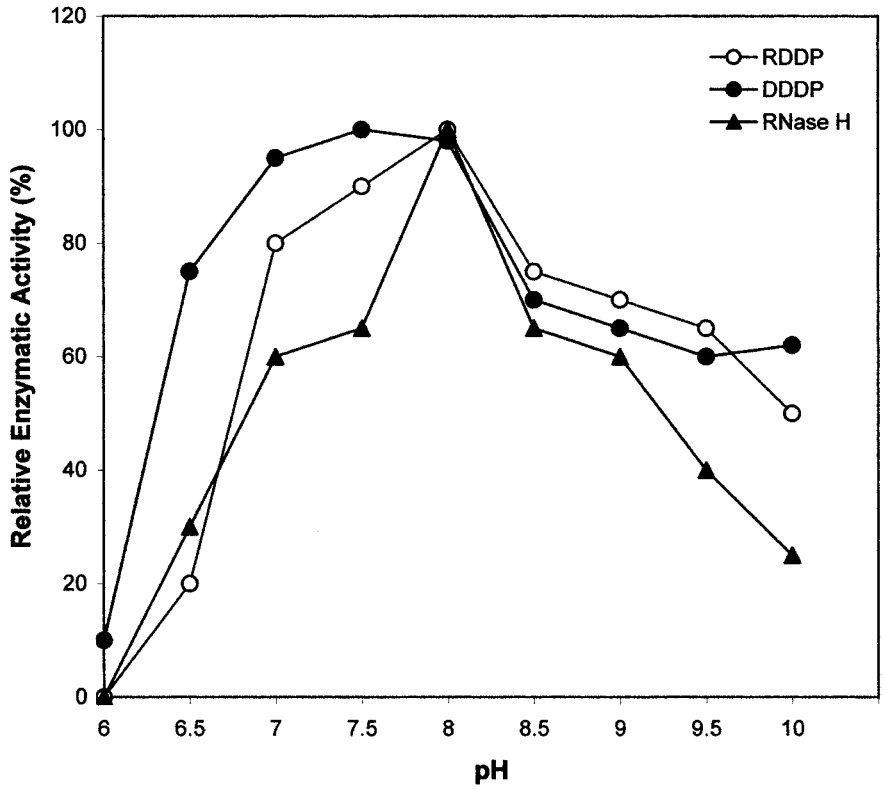


FIG. 3. The effects of pH on the catalytic activities of BLV RT. The RDDP, DDDP, and RNase H assays were conducted as described under Materials and Methods by varying the pH values. For reactions performed at pH 6.0–6.5, 25 mM sodium acetate was used; for pH 6.5–9.0 25, mM Tris–HCl was used; 25 mM glycine-NaOH was used for pH values 9.0–10. Enzymatic values are expressed as percentages of maximal activity observed. (○) RDDP, (●) DDDP, (▲) RNase H

ers. The K_m values for the DDDP activity were calculated with activated DNA (prepared as described under Materials and Methods) for both dGTP and dTTP. It is apparent from Table 3 that the K_m values calculated for BLV RT are comparable with the values reported for the RTs of MMTV, EIAV, HIV-1, and HIV-2 (Hizi *et al.,* 1991b; Rubinek *et al.,* 1994; Taube *et al.,* 1998).

TABLE 3

The K_m Values for dNTPs and Template-Primers Calculated for BLV RT in the DNA Polymerase Reactions

	K_m (μM)		K_m (μg/ml)	
Activity	dTTP	dGTP	Poly(rA)$_n$ · oligo(T)$_{12-18}$	Poly(rC)$_n$ · oligo(dG)$_{12-18}$
RDDP	9.4 ± 1.4	9.8 ± 1.0	4.8	12.7
DDDP	2.0 ± 0.6	1.9 ± 0.5	N.A.	N.A.

Note. The K_m values were determined from the double-reciprocal plots of the varied substrate concentrations against the initial rates of DNA synthesis. The plots were computer-generated by linear regression analyses. Assays were conducted as described under Materials and Methods. For RDDP activity, dTTP was assayed with poly(rA)$_n$ · oligo(dT)$_{12-18}$, whereas dGTP was assayed with poly(rC)$_n$ · oligo(dG)$_{12-18}$. The template-primer used for the DDDP activity was activated DNA. N.A., not applicable.

Inhibition of the DNA polymerase activities by nucleoside analogues

All RTs exhibit some sensitivity to nucleoside analogue inhibitors that serve as DNA chain terminators. This sensitivity varies, depending on the RT studied, on the mutants of every given RT and on the inhibitors tested. Thus such drugs are being used successfully for the treatment of HIV infections in humans, e.g., 3′ azido-2′,3′ dideoxythymidine (AZT), 2′,3′-dideoxyinosine (ddI), and (−)-2′-deoxy-3′-thiacytidine (3TC). These nucleoside analogues are potent inhibitors of the RTs of HIV-1 and HIV-2 (De Clercq, 1995). There are, however, frequent emergences of drug resistance to these inhibitors by mutating these RTs (Skalka and Goff, 1993; Coffin *et al.,* 1997). We have assayed BLV RT in the presence of the chain terminator ddGTP ddTTP and AZTTP, which are known to be potent competitive inhibitors of the RTs of HIV-1, HIV-2, and MMTV. It is apparent that these analogues inhibit both the RDDP and DDDP activity of BLV RT with the former activity much more sensitive to these compounds (Table 4). However, when the IC_{50} values calculated for BLV RT are compared with those of the HIV-1, HIV-2, and MMTV, it is clear that this novel recombinant enzyme is substantially less sensitive to the inhibitors. In this regard, BLV RT is closer to the more

TABLE 4

Inhibition of the RDDP and DDDP Activities of BLV RT by dNTP Analogues

Enzymatic activity	Inhibitor (IC_{50} values in nM)		
	ddGTP	ddTTP	AZTTP
RDDP	20 ± 4	86 ± 2	80 ± 4.3
DDDP	3900 ± 200	860 ± 61	1050 ± 90

Note. The increasing dNTP analogs were tested by using increasing concentrations of each chain terminator—RDDP activity was assayed with either poly(rA)$_n$ · oligo(dT)$_{12-18}$ and dTTP (for the inhibitors dTTP and AZTTP) or poly(rC)$_n$ · oligo(dG)$_{12-18}$ and dGTP for the analogue ddGTP. DDDP was assayed with activated DNA and all four dNTPs for all three analogues. In all cases, we have observed competitive inhibitions. The concentrations of inhibitors leading to a reduction of 50% in the initial polymerase activity (IC_{50} values) were calculated from the inhibition curves. The IC_{50} values were calculated each from at least three independent experiments.

resistant RT of equine infectious anemia virus (EIAV), which despite being a lentivirus, shows distinct differences from HIV RTs (Hizi *et al.,* 1991b; Rubinek *et al.,* 1994; Taube *et al.,* 1998).

Inhibition of the catalytic functions of BLV RT

The effects of NaCl and KCl on the enzymatic activities of BLV RT. Most RTs studied require NaCl or KCl at final concentrations of 50–100 mM for their optimal activities of both DNA polymerase and RNase H. We have shown recently that MMTV RT is quite sensitive to these salt concentrations, and the highest levels of activities are obtained when assayed with no monovalent cations present (Taube *et al.,* 1998). Therefore it was of interest to test also the effects of NaCl and KCl on BLV RT. The results shown in Fig. 4A, show that BLV RT is quite sensitive to the presence of NaCl and KCl in all catalytic functions. Of the DNA polymerase activities, the RDDP activity is slightly more susceptible (with an IC_{50} value of ~60 mM) than the DDDP activity (IC_{50} of ~90 mM). The RNase H is sensitive as is the RDDP. In addition there is very little difference between the response to NaCl compared with KCl. In all, BLV RT is quite similar to MMTV RT in its high susceptibility to salts.

Involvement of cysteine residues in the enzymatic activities of BLV RT. Most RTs studied so far were shown to be sulfhydryl-requiring enzymes, namely, reduced cysteine groups participate in forming the functionally active catalytic sites (Skalka and Goff, 1993). The exception to this rule are the RTs of HIV-1 and HIV-2, where the DNA polymerase functions are fully resistant to thiol reagents (although the RNase H activity is highly sensitive to these reagents). In the present study, we have tested the sensitivity of the enzymatic activities of BLV RT to the sulfhydryl-specific reagent *N*-ethylmaleimide (NEM). Previous studies have shown that the response of RTs to NEM is similar to the response to other thiol-specific reagents: consequently, NEM can serve as a typical thiol reagent (Hizi *et al.,* 1991b, 1992). The results described in Fig. 4B show that all activities of BLV RT are inhibited by NEM. The IC_{50} value for RDDP activity is ~0.6 mM and for the DDDP function is ~1.5 mM NEM. As found previously for other RTs, the RNase H activity is more sensitive to NEM than the DNA polymerase activities, i.e., the IC_{50} value is ~0.15 mM. These results strongly suggest that reduced cysteine groups are essential for all activities of BLV RTs. This conclusion could be expected in view of the amino acid sequence of BLV RT. The 584-residue-long polypeptide of the recombinant BLV RT contains five cysteine residues.

Effects of the Zn^{+2} chelator orthophenanthroline (OP) on BLV RT. Many DNA polymerases as well as all RTs studied were documented to be Zn^{+2} metalloenzymes because they are inhibited by the specific chelator OP. It is apparent that BLV RT is also sensitive to OP, with RNase H function less sensitive than the DNA polymerase activity (where the RDDP activity is more susceptible than the DDDP function; see Fig. 4C). Similar patterns of inhibition (in the order of sensitivity of RDDP > DDDP > RNase H) were observed for other recombinant RTs (i.e., those of HIV-1, HIV-2, EIAV, and MMTV), suggesting a similar involvement of Zn^{+2} in their catalytic activities (Hizi *et al.,* 1991b; Rubinek *et al.,* 1994; Taube *et al.,* 1998). In fact, the RNase H activity of MMTV, HIV-1, and HIV-2 RTs is fully resistant to OP, up to ~6 mM OP. This led us to the suggest that these RTs bind at least two Zn^{+2} atoms. The first one, which is more accessible to OP, is involved in the DNA polymerase activity. The other atom, which is mainly involved in the RNase H function, binds the protein more tightly and, consequently, is chelated at higher OP concentrations (Hizi *et al.,* 1991b; Taube *et al.,* 1998). It is possible that this general scheme is also relevant to BLV RT.

DISCUSSION

In contrast to the reverse transcriptases of lentiviruses (i.e., HIV-1, HIV-2, and EIAV), type C retroviruses (i.e., MLV), and type B retroviruses (i.e., MMTV), the recombinant expression and structure-function relationship of the RTs of the BLV/HTLV subgroup of retroviruses were never studied until very recently. The present paper describes the expression and biochemical analyses of the BLV enzyme. The strategy for constructing the BLV RT-coding gene, described in the present publication, assumes that this RT is a transframe protein (Fig. 1A). The amino terminus of BLV RT is adjacent to the carboxyl terminal residue of the mature viral protease (Rice *et al.,* 1985; Yoshinka *et al.,* 1986) and is coded by the last 26 codons of the *pro* gene open reading frame (followed by the *pol* gene codons). This implies that a 26-amino-acid residue peptide identical to the amino terminal 26 resi-

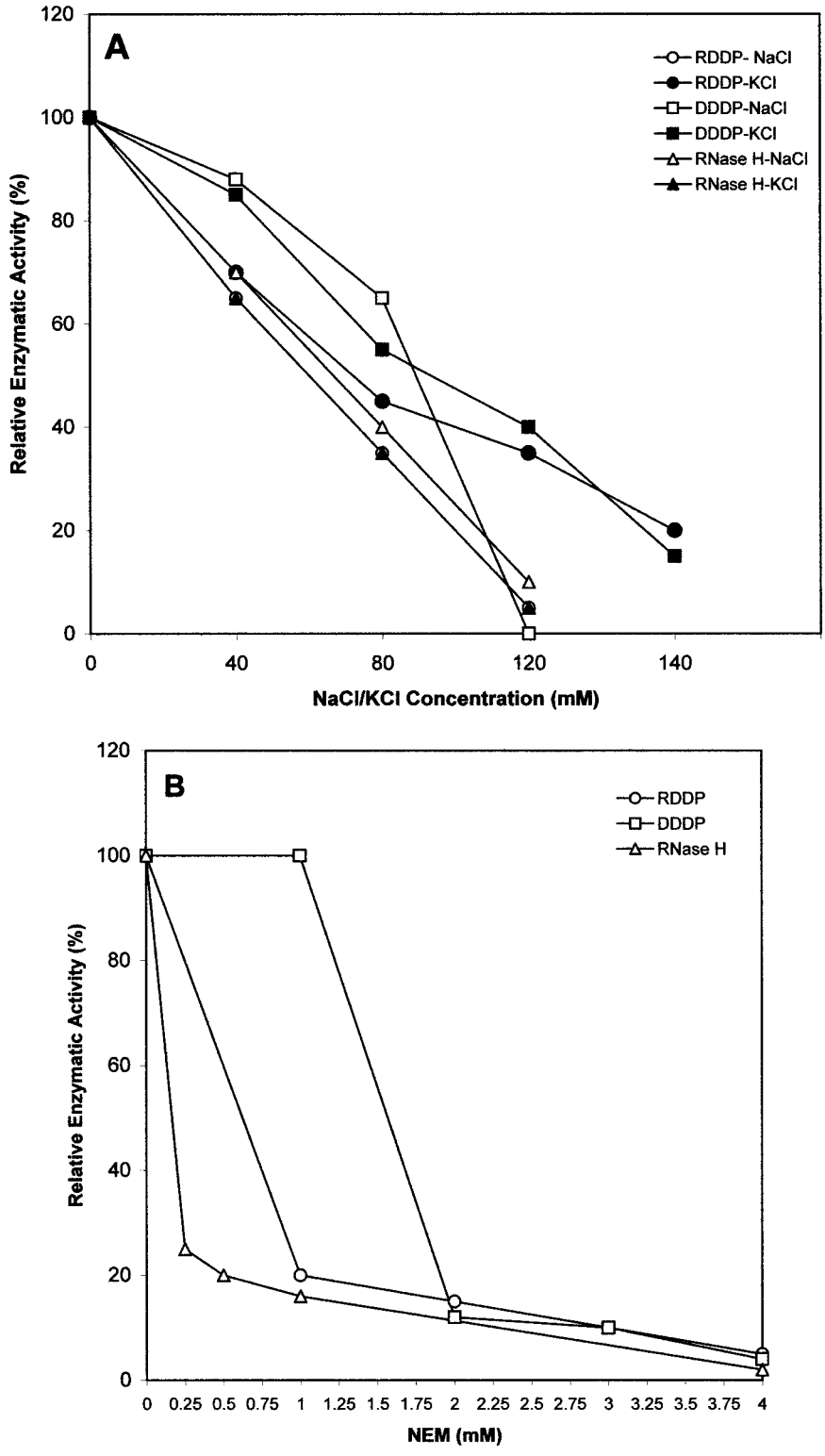


FIG. 4. Effects of NaCl, KCl, NEM, and the zinc chelator OP on the catalytic activities of BLV RT. The various enzymatic activities of BLV RT were tested with increasing concentrations of NaCl or KCl (A), NEM (B), or OP (C). The RT activities were assayed as described under Materials and Methods in the presence of increasing concentrations of NaCl, KCl, NEM, or OP and presented as the enzymatic activities relative to controls with no inhbitors present. When NEM was used, the RT was preincubated with NEM on ice for 30 min before adding the appropriate substrate and assaying the specified activities. (A) Open symbols are for NaCl and closed ones for KCl: ○, ●, RDDP; □, ■, DDDP; △, ▲, RNase H. (B and C) ○, RDDP; □, DDDP; △, RNase H.

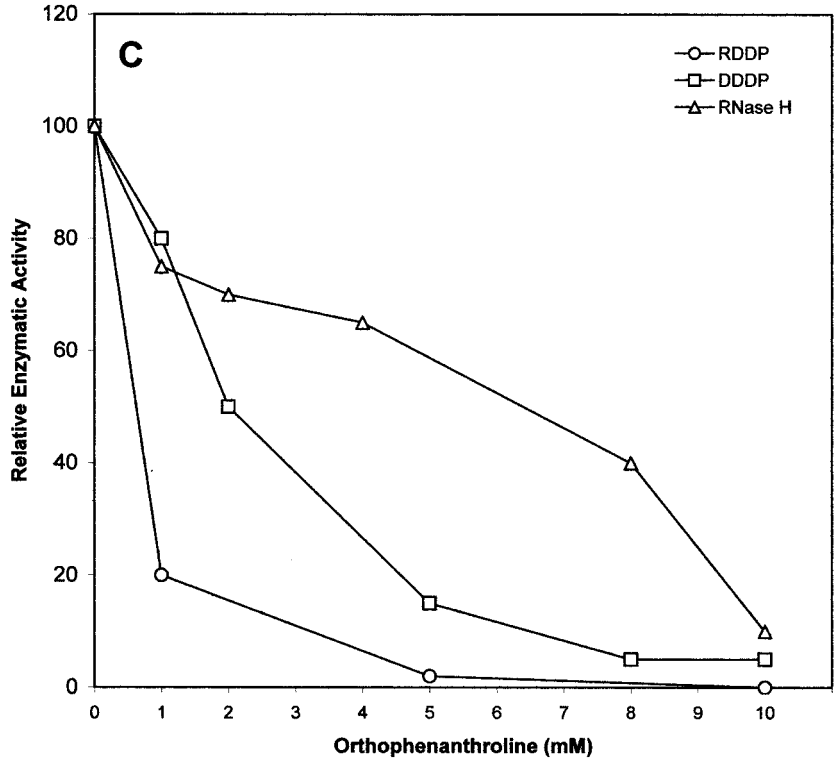


FIG. 4—*Continued*

due sequence of BLV RT is released after the Gag-Pro polyprotein precursor is cleaved by the viral protease in the maturating virions. Based on this supposition, we postulate that such a peptide is likely to be found in virions of BLV. It is interesting to note that this sequence is proline rich; i.e., 7 of the 26 residues are prolines, including two sets of double prolines (Fig. 1A). Because prolines are known to terminate α helixes and promote turns, the presence of the prolines in this amino terminal sequence (and, more importantly, the presence of two sets of double prolines) might be very significant for the proper folding and enzymatic properties of BLV RT. We plan to study the involvement of these proline clusters in the structure-function relationships in BLV RT as done by us previously for the highly conserved two sets of double prolines found in HIV-1 RT (Hizi and Shaharabany, 1992). In addition we plan to study the virus-derived BLV RT to check whether it is also a transframe protein and how similar is the recombinant protein to the viral RT.

When this research was near its completion, two very recent reports were published on the expression of RT of HTLV-1, which is related to BLV. In the first one, the enzymatically active HTLV-1 RT expressed in bacteria was coded for by the *pol* gene of the MT-2 isolate, starting with proline 33 in the *pol* reading frame. The codon for this proline is located within the putative *pro-pol* frameshift site (Owen *et al.,* 1998). In the second report, the amino terminus of the HTLV-1 RT is derived from codons of the *pro* gene followed by sequences of the *pol* gene after adding an extra C in the putative frameshift site to account for the frameshifting event (Trentin *et al.,* 1998). This strategy of gene construction is similar to that employed for BLV RT in the present study with the exception that the *pro*-derived sequences do not extend in the case of HTLV-1 RT until the *pro* gene stop codon (as done in our study) because the putative frameshift site in HTLV-1 is located upstream to this termination codon. In the latter study of HTLV-1 RT expression, the amino terminus for the RT is, as in our study, adjacent to the carboxyl terminus of the protease; however, since Trentin *et al.* assumed that the HTLV-1 RT sequence is similar to that of the RT of avian sarcoma virus (ASV). They have constructed an oligomeric structure of the RT, i.e., RT/RT–IN (which resembles the α/β subunits, respectively, of ASV RT) and claim to have obtained, as solely enzymatically active RT form, the α_3/β tetramer. This is apparently a molecular arrangement never encountered before in RTs (Skalka and Goff, 1993; Coffin *et al.,* 1997; Hizi and Joklik, 1997). Taken together, there are substantial discrepancies between the two published reports on recombinant HTLV-1 RT. It is apparent from our study of BLV RT that the amino acid sequence requirements and molecular arrangements of the recombinant BLV RT are substantially different from those of both HTLV-1 RT studies. These unique differences for relatively close RTs should be helpful to future studies designed to study structure-function relationships of these RTs.

The biochemical studies of the partially purified recombinant BLV RT reveal a similarity to other previously studied RTs. The enzyme possesses all the known activities of RTs and like most RTs prefers Mg^{+2} over Mn^{+2} as divalent cations required for the catalytic activities. A comparison between the biochemical properties of the recombinant BLV RT and the RT activity in BLV virions (Gilden *et al.,* 1975; Mamoun *et al.,* 1981; Demirhan *et al.,* 1996) is not straightforward because only a few biochemical features were studied for the RT in crude viral lysates. All BLV RT preparations prefer Mg^{+2} over Mn^{+2} for catalysis. In one study (Demirhan *et al.,* 1996), the molecular weight of the crude viral RT was determined to be of ~80 kDa, and the enzyme was shown to have some preference for poly(rC) · oligo(dG) over poly(rA) · oligo(dT) as substrates for the RDDP activity. Because of the relatively low accuracy of the method employed for the molecular weight determination of the viral RT, it is fair to conclude that the figure of ~80 kDa is not very different from the molecular weight of the monomeric recombinant BLV RT obtained in our study (~65 kDa). However, the substrate preference is clearly different from the one found in our study, where poly(rA) · oligo(dT) is better than poly(rC) · oligo(dG) (see Table 2). This dissimilarity should be further investigated.

There are several unique features that set the novel recombinant BLV RT apart from most other RTs studied so far. As discovered recently for MMTV RT (Taube *et al.,* 1998), BLV RT is capable of being catalytically active as a monomer even after binding to nucleic acids. Unlike MLV RT, such a binding does not convert the BLV RT protein to homodimers. This feature cannot be attributed to the recombinant expression system because most RTs expressed by us using the same system, (i.e., those of HIV-1, HIV-2, and EIAV) were dimers (Hizi *et al.,* 1988, 1991a; Shaharabany *et al.,* 1993), whereas MLV RT was a monomer (Hizi and Hughes, 1988). Moreover as mentioned above, a study on virus-derived BLV RT suggested a molecular weight of ~80 kDa, certainly closer to monomers (of ~65 kDa) than to potential dimers (of ~130 kDa) (Demirhan *et al.,* 1996). The sensitivity to salts, NaCl and KCl, is also quite unique to BLV RT. Here again this singular property was also reported for MMTV RT (Taube *et al.,* 1998). The question that arises from both outlined unique properties obviously is whether there is any connection between the monomeric behavior of the active RT and the fact that its catalytic activities are salt sensitive. A mechanistic study toward addressing this question might be highly important in explaining the dimerization process in retroviral RTs and its involvement in catalysis. Recent studies have tried to localize amino acid sequences responsible for dimerization and to analyze the kinetics of the polymerization process in HIV RT and EIAV RT (Divita *et al.,* 1995; Wohrl *et al.,* 1997; Souquet *et al.,* 1998). However, it is not clear yet how this process affects enzymatic activity. Future crystal studies of BLV or MMTV RTs should shed light on the folding of monomeric RTs in comparison with the folding patterns of dimeric RTs, such as HIV-1 RT or MLV RT (Kohlstaedt *et al.,* 1992; Georgiadis *et al.,* 1995; Jacobo Molina *et al.,* 1993; Huang *et al.,* 1998).

The purification pattern of the recombinant RT from the bacterial lysates (Table 1) suggests removal of strong inhibitors because the overall activity goes up throughout the purification steps. We have observed a similar phenomenon while purifying recombinant MMTV RT (Taube *et al.,* 1998). Interestingly, we have never encountered such an increase in the total activity while purifying recombinant RTs of HIV-1, HIV-2, or EIAV from similar bacterial extracts (unpublished data), suggesting a somewhat specific inhibition of only MMTV and BLV RTs.

The sensitivity of RTs to nucleoside analogues is well established, and several of the most common drugs against HIV infections in humans, such as AZT, 3TC, and ddI, take advantage of the high susceptibility of HIV RT to such drugs. Generally speaking, the RTs can be divided into two major groups depending on the extent of this sensitivity. The first one includes the highly sensitive RTs of HIV-1, HIV-2 feline immunodeficiency virus (FIV) and MMTV (Hizi *et al.,* 1991; De Clercq, 1995; North and LaCasse, 1995; Taube *et al.,* 1998). The second group of the less sensitive RTs include RTs of mammalian type C retroviruses (such as MLV) and EIAV (Hizi *et al.,* 1988; Rubinek *et al.,* 1994; Coffin *et al.,* 1997). The present study indicates that BLV RT belongs to the second group. There are numerous studies on the involvement of different amino acid residues of HIV-1 and HIV-2 RTs in this sensitivity to drugs (e.g., Skalka and Goff, 1993; Perach *et al.,* 1995; Coffin *et al.,* 1997). All of them were performed by lowering the high sensitivity of the wild-type RTs by modifying selective residues, thus leading to drug resistance. However, there are no extensive studies on converting wild-type RTs with low sensitivity levels to variants with high sensitivities by using a similar mutagenesis approach. BLV RT can serve for such structure-function mutagenesis studies in the future.

The sensitivity of RTs to chemical modifying compounds such as sulfhydryl reagents is also useful in studying the biochemistry of these enzymes. All RT studied show some sensitivity to NEM. HIV-1 and HIV-2 RTs are the only ones that show NEM-resistant DNA polymerase activities and, yet, have a highly sensitive RNase H activity (Hizi *et al.,* 1988). Interestingly, none of the two or three cysteine residues present in these RTs (in HIV-1 RT, and HIV-2 RT, respectively) are located in the RNase H subdomain. All other RTs studied show a sensitivity to NEM in both DNA polymerase and RNase H functions. BLV RT belongs to this group of RTs (Fig. 4B). Still, as in HIV RTs, all five cysteines, of BLV RT are located outside the putative RNase H subdomain. Therefore it is not surprising that the DNA polymerase of BLV RT is NEM sensitive. It is not fully clear, however, why the RNase H

activity is sensitive. As already speculated for HIV RTs, this certainly reflects a tight interplay between the DNA polymerase and RNase H domains of BLV RT as well. Future three dimensional studies of BLV RT as well as other RTs will probably resolve this question.

MATERIALS AND METHODS

Plasmids and enzymes

The RTs used in this study are recombinant enzymes expressed in *E. coli* DH5α and purified from the bacterial extracts. The BLV RT coding gene was derived from a pBR322 plasmid containing a BLV provirus (as *Sac*I insert introduced into the *Pst*I site of the plasmid), which was a generous gift of Dr. L. Willems of the Faculte' des Sciences Agronomiques in Gembloux, Belgium. The sequence of this BLV provirus was published (Rice *et al.,* 1985), and the numbering of the DNA sequences was done according to this published sequence. The RT-encoding gene was subcloned into the expression plasmid pUC12N, which differs from the original pUC12 in that the sequence flanking the *lacZ* initiator ATG codon was modified to accommodate an *Nco*I restriction site. This plasmid was already used by us for a constitutive overexpression of a variety of recombinant RTs in bacteria (Hizi *et al.,* 1988, 1991a; Hizi and Hughes, 1998; Taube *et al.,* 1998). Recombinant HIV-1 RT was expressed in bacteria and purified as described (Hizi *et al.,* 1988; Clark *et al.,* 1990).

Construction of BLV RT expression plasmids

All BLV-related DNA segments that were incorporated into the pUC12N expression plasmid were generated by PCR, using thermostable Vent DNA polymerase (NEB) and the BLV proviral DNA as a template. The sequences and localization on the BLV genome, the synthetic DNA primers used in the different PCR reactions, as well as the proteins expressed, are all described under Results. The DNA segments were digested with *Nco*I and *Hin*dIII and ligated into pUC12N plasmids, which were then introduced into competent cells of the DH5α strain of *E. coli* by transformation. Bacterial transformation, growth, the analysis of the bacterial proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and the enzymatic assay for RDDP in bacterial lysates were all described in detail previously (Hizi and Hughes, 1988; Hizi *et al.,* 1988, 1991a).

Assays of the DNA polymerase and RNase H activities

All enzymatic reactions were performed basically as described previously (Hizi *et al.,* 1991b; Rubinek *et al.,* 1994; Taube *et al.,* 1998). RDDP activity was routinely assayed by following the poly(rA)$_n$ · oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP into DNA. Assays were conducted, unless otherwise stated, in 25 mM Tris–HCl, 8 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 5 μg/ml poly(rA)$_n$ · oligo(dT)$_{12-18}$ (Pharmacia), 5 μM [^{3}H]dTTP (specific radioactivity 2000–4000 CPM/pmol) at a final pH of 8.0. Incubation was performed for 30 min at 37°C (or 10 min for the kinetic studies). In several experiments, we have used for assaying the RDDP activity poly(rC)$_n$ · oligo(dG)$_{12-18}$ and [^{3}H] dGTP replacing poly(rA)$_n$ · oligo(dT)$_{12-18}$ and [^{3}H]dTTP, respectively. The DDDP activity was assayed under similar conditions with herring sperm activated DNA as the template-primer, [prepared by a limited digestion with bovine pancreatic DNase I (Sigma), as described in detail previously (Shaharabany and Hizi, 1991)], suppliment by all four dNTPs (of which only one, dTTP, was radioactively labeled). RNase H activity was assayed by measuring the hydrolytic release of the radioactively labeled molecules from [^{3}H]poly(rA) (Amersham) annealed to poly(dT)$_n$ (Pharmacia) into the trichloroacetic acid-soluble (TCA) fraction. The assay was performed at pH 8.0 in a final volume of 100 μl containing 50 mM Tris–HCl, 50 mM KCl, 8 mM $MgCl_2$, 2.5 mM DTT, and ~100 pmol of [^{3}H] poly(rA)$_n$ · poly(dT)$_n$. The enzymatic activities were defined as follows: one unit of DNA polymerase activity is the amount of enzyme catalyzing the incorporation of 1 pmol of dNTP into DNA product after 30 min at 37°C under standard assay conditions. One unit of RNase H activity is the amount of enzyme catalyzing the release of 1 pmol of AMP in 30 min at 37°C under the assay conditions.

Protein purification of BLV RT

We have transferred the RT coding DNA insert into the expression plasmid pUC12N6H, which codes for a six-histidine tag at the amino terminus of the recombinant protein. The transformed bacteria were grown at 37°C in an NZYM medium containing 100 μg/ml ampicillin. After ~4 h of growth, the *E. coli* culture was used to inoculate a freshly-prepared 0.5-liter culture of the same medium supplemented by 50 μg/ml ampicillin. This culture was grown for 12–16 h at 37°C, and the cells were harvested by centrifugation at 12,000 *g* for 15 min at 4°C. The pelleted cells were washed three times with ice-cold TNE buffer (10 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, pH 8.0) and stored at −70°C until the purification.

Preparation of crude lysates. All purification steps were conducted at 4°C. The frozen cells were lysed for 15 min with lysis buffer (50 mM NaCl, 20 mM HEPES–NaOH, 1 mM PMSF, pH 7.0, containing 0.5 mg/ml lysozyme at the ratio of 2 ml buffer per gr of wet bacteria). After 15 min on ice, NaCl was added to a final concentration of 0.5 M and sonicated repeatedly on ice for a total of 5 min. Then polyethylene glycol at a final concentration of 0.3% was added and after stirring 15 min on ice. The cell debris and nucleic acids were precipitated by centrifuging for 40 min at 8000 *g* at 4°C.

Ni^{2+} nitriloacetic acid (Ni–NTA) agarose affinity chromatography. The supernatant was collected and diluted 1:1 with loading buffer (20 mM HEPES–NaOH, 0.5M NaCl, final pH 7.8). The Ni–NTA agarose column (Qiagen) was prewashed thoroughly with the same equilibration buffer. The bacterial extracts were loaded on the column and washed extensively with the same buffer. The column was further washed with 50 mM ammonium acetate, 0.3 M NaCl, pH 6.0, and the histidine-tagged protein was eluted with the same buffer containing a 0–0.5 M linear gradient of imidazole (adjusted to pH 6.0). Fractions were collected, assayed for RDDP activity and analyzed by SDS–PAGE. Peak fractions were pooled and dialyzed extensively against 20 mM Tris–HCl,10% (v/v) glycerol, 25 mM NaCl, 0.1% (v/v) Triton X-100, 2 mM DDT, and 1 mM EDTA (final pH 7.5).

Carboxymethyl (CM)–Sepharose cation exchange chromatography. The column was pre-equilibrated with CM buffer [10 mM sodium phosphate, 2 mM DTT, 10% (v/v) glycerol, pH 7.0] and then loaded with the dialyzed material, followed by extensive washing with the same buffer. The RT was eluted with the same buffer containing a 0–0.5 M linear gradient of NaCl in the same buffer. Peak reactions were pooled and dialyzed against 20 mM Tris–HCl (pH 8.0), 25 mM NaCl, 50% (v/v) glycerol, 0.2% (v/v) Triton X-100, 2 ml mM DTT, and 1 mM EDTA. The purified RT was divided into aliquots and stored for long periods at −70°C and for routine analyses at −20°C.

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