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Rice tungro bacilliform virus encodes reverse transcriptase, DNA polymerase, and ribonuclease H activities

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ABSTRACT **Rice tungro bacilliform virus (RTBV) is a newly described badnavirus and proposed member of the plant pararetrovirus group. RTBV open reading frame 3 is predicted to encode a capsid protein, protease (PR), and reverse transcriptase (RT) and has the capacity to encode other proteins of as yet unknown function. To study the possible enzymatic activities encoded by open reading frame 3, a DNA fragment containing the putative PR and RT domains was used to construct the recombinant baculovirus PR/RT-BBac. *Trichoplusia ni* insect cells infected with PR/RT-BBac were used in pulse-labeling experiments and demonstrated synthesis of an 87-kDa polyprotein that corresponds in molecular mass to that predicted from the PR/RT DNA coding sequence. The 87-kDa polyprotein was processed with concomitant accumulation of 62-kDa (p62) and 55-kDa (p55) proteins. Amino-terminal sequencing of p62 and p55 determined that they mapped to the PR/RT domain and shared common amino termini. p62 and p55 were purified and exhibited both RT and DNA polymerase activities using synthetic primer/template substrates. Only p55 had detectable ribonuclease H activity, an activity intrinsic to all reverse transcriptases studied to date. Characterization of the RTBV RT provides a biochemical basis for classifying RTBV as a pararetrovirus and will lead to further studies of these proteins and their role in virus replication.**

Rice tungro bacilliform virus (RTBV) is one of two viruses causally responsible for rice tungro disease in Asia; the other is rice tungro spherical virus (1). The structure and sequence of the RTBV double-stranded DNA genome and predicted amino acid sequence led to the proposal that RTBV is a pararetrovirus (2); this was based upon similarities with other plant pararetroviruses.

The replication cycle of plant pararetroviruses is initiated by the synthesis of a terminally redundant genome-length transcript under the control of the viral promoter. This transcript, when primed by a host cell tRNA, is used as a template by the viral-encoded reverse transcriptase (RT) for synthesis of the minus-strand DNA. Ribonuclease H (RNase H) degrades the RNA in the RNA·DNA hybrid, leaving one or more specific RNA fragments, which are used by the RT, now acting as a DNA-dependent DNA polymerase, to prime synthesis of the plus-strand viral DNA, completing the circular double-stranded DNA genome (for reviews, see refs. 3 and 4).

Several features of RTBV provided further evidence that it belonged to the pararetrovirus group. First, RTBV-infected rice plants accumulate a terminally redundant genome-length transcript complementary to the minus strand of the RTBV genome (2), thus providing key evidence for an RNA intermediate in the viral replication cycle. Second, a transcriptional promoter that led to the synthesis of this transcript was identified (5). Third, RTBV was predicted, through amino acid sequence similarities with other plant pararetroviruses, to encode a RT (2). We initiated studies to characterize the proposed RT domain in open reading frame (ORF) 3 of RTBV to clarify the mode of RTBV replication.

The only other plant pararetrovirus RT that has been studied in detail is that of cauliflower mosaic virus (CaMV) (6). ORF V of CaMV was determined to encode a 78-kDa polyprotein that in *in vitro* reactions was processed by the protease (PR) in the polyprotein to release the RT domain, though an assay for RT activity was not done (7). When ORF V was expressed in yeast, RT activity was detected in fractionated cell lysates, but evidence for proteolytic processing of the polyprotein was not presented (8). In addition, while it has been proposed that the CaMV RT contains an RNase H domain (9), there is no corresponding biochemical evidence (10).

Since RTBV is a phloem-limited virus, only a fraction of the cells in a rice plant become infected (11). Therefore, a heterologous system was chosen for overexpression of the RTBV RT. Qu *et al.* (2) proposed that ORF 3 encoded a polyprotein containing a capsid protein, PR, and RT. In the current study, a fragment of the RTBV genome, containing the putative PR and RT domains, was expressed using a recombinant baculovirus in insect cells. Here we demonstrate that insect cells expressing the RTBV PR/RT ORF synthesize and process the polyprotein, with the resulting accumulation of polypeptides possessing RT, DNA polymerase, and RNase H activities.

MATERIALS AND METHODS

Constructs. The full-length RTBV clone pBSR63A (2) was digested with *Xba* I and *Nhe* I, and a 2.7-kb DNA fragment containing the predicted PR and RT domains (nt 3735–6486) was cloned into pBluescript KS$^+$ (Stratagene) behind the T7 RNA polymerase promoter (Fig. 1). The resulting plasmid, pBS-PR/RT, was digested with *Xba* I and *Bam*HI, and the 2.7-kb DNA fragment, which contains an ATG codon at the 5′ end, was ligated downstream of the polyhedrin promoter in the baculovirus transfer vector pBlueBac II (Invitrogen), resulting in pBBac-PR/RT. pBS-PR/RT was also digested with *Eco*RV and *Bam*HI (Fig. 1), and the resulting 2-kb fragment was cloned into the *Escherichia coli* expression vector pT7-7 (12). The resulting plasmid pT7-ΔPR contained RTBV nt 4536–6486 in-frame with the pT7-7 ATG codon. Transcription of the ΔPR sequence is directed by the T7 RNA polymerase promoter.

***In Vitro* Transcription and Translation of the PR/RT Domain.** The plasmid pBS-PR/RT (Fig. 1) was linearized using

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Abbreviations: RT, reverse transcriptase; PR, protease; RTBV, rice tungro bacilliform virus; ORF, open reading frame; HIV, human immunodeficiency virus; CaMV, cauliflower mosaic virus; ssDNA, single-stranded DNA.

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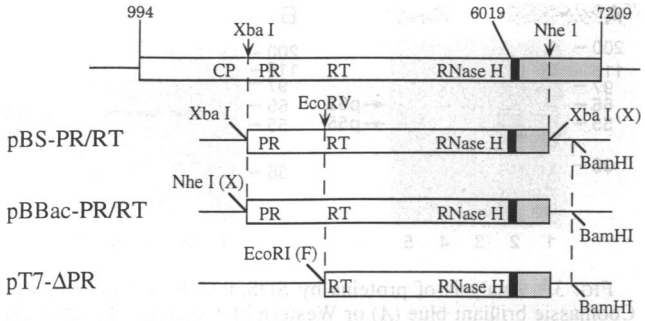

FIG. 1. ORF 3 of RTBV (top schematic) and derived fragments used to construct various vectors (indicated on left). The intergenic region linking ORF 3 and ORF 4 (20 nt) is black; ORF 4 is shaded. Numbers indicate nucleotides in the RTBV genome (2). Location of coat protein (CP) and predicted consensus amino acid sequences for PR, RT, and RNase H are indicated. Arrows denote restriction sites in the RTBV sequence; all other restriction sites are contained in the respective vector (see *Materials and Methods*). X, restriction site lost after ligation; F, prior to ligation the restriction site was filled in using Klenow polymerase and dNTPs.

*Bam*HI and then transcribed *in vitro* using T7 RNA polymerase (New England Biolabs). The resulting ≈2.8-kb mRNA was translated using reticulocyte lysate supplemented with [^{35}S]methionine (Amersham) according to the manufacturer's recommendations (GIBCO/BRL). A sample was run in SDS/PAGE, stained and destained, treated with Amplify (Amersham), dried, and exposed to x-ray film. Molecular size markers included β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), and lactate dehydrogenase (36.5 kDa).

Expression of pT7-ΔPR in *E. coli* and Anti-ΔPR Antiserum Production. *E. coli* (DH5αF′) was transformed with pT7-ΔPR, and ampicillin-resistant cells were grown to OD_{600} = 0.6. The culture was infected with mGP1-2, a recombinant M13 phage carrying the T7 RNA polymerase gene (12), and after 3 hr the cells were pelleted. The 57-kDa ΔPR protein accumulated in inclusion bodies, thus simplifying its purification (13). The ΔPR protein was further purified by SDS/PAGE (14) and electroblotted to nitrocellulose (15). The area of the filter containing the ΔPR protein was ground into a fine powder, mixed with adjuvant, and used to prepare antibody in New Zealand White rabbits as described (16).

Recombinant Baculovirus. The growth of *Spodoptera frugiperda* (Sf9) insect cell cultures and propagation of recombinant baculovirus were carried out as described (17). *Trichoplusia ni* (TN5) insect cells (High Five; Invitrogen) were treated in the same manner as Sf9 cells except that they were grown in EX-CELL 400 medium (JRH Biosciences, Lenexa, KS).

Either the original plasmid pBlueBac II or pBBac-PR/RT was cotransfected with linearized *Autographa californica* nuclear polyhedrosis virus DNA into Sf9 cells using the cationic liposome method according to the manufacturer's instructions (Invitrogen). Recombinant baculoviruses were plaque purified. Viral DNA was extracted from budded virions and used in polymerase chain reactions with primers complementary to sequences 5′ and 3′ to the cloning site to confirm that RTBV sequences were retained. Recombinant baculovirus containing no PR/RT insert (ΔPH-BBac) or the PR/RT insert (PR/RT-BBac) was used in all further studies.

Preparation of Baculovirus-Infected Cell Lysates. TN5 insect cells were grown in tissue culture flasks for all experiments. The multiplicity of baculovirus infection was 10. At 72 hr postinfection the cells were harvested, rinsed in PBS, and resuspended at a density of 1×10^7 cells per ml in lysis buffer containing 50 mM Tris·HCl (pH 7.5), 20 mM dithiothreitol, 150 mM NaCl, 0.5% (vol/vol) Nonidet P-40, 2 mM EDTA, 10% (vol/vol) glycerol, leupeptin at 1 μg/ml, phenylmethylsulfonyl fluoride at 200 μg/ml, and aprotinin at 50 μg/ml. Cells were incubated on ice for 15 min and then pelleted at ≈3000 × *g* for 10 min. The supernatant was collected and recentrifuged at 120,000 × *g* for 1.5 hr. Supernatants were frozen in liquid N_2 and stored at −80°C.

Pulse-Labeling of Insect Cells. ΔPH-BBac or PR/RT-BBac-infected TN5 cells were pulse-labeled with [^{35}S]methionine (Amersham) for 15 min at 44 hr postinfection as described (17). The cells were lysed as described above except that pepstatin A (1.5 μg/ml) was included in the lysis buffer. Cell lysates were centrifuged at 12,000 × *g* for 5 min; supernatants were frozen in liquid N_2 and stored at −80°C. Protein samples were analyzed by SDS/PAGE, after which the gel was stained and destained, treated with Amplify, dried, and exposed to x-ray film.

Protein Purification. Nine milliliters of the TN5 cell lysate (3 mg of protein per ml) was loaded onto a 5-ml (bed volume) column containing single-stranded DNA (ssDNA)-cellulose (Pharmacia) and eluted with a linear 25–300 mM NaCl gradient in buffer A (50 mM sodium phosphate, pH 7.4/25 mM NaCl/2 mM EDTA/10% glycerol). The peak fractions of RT activity were collected and applied to a FPLC Mono S 5/5 column (Pharmacia). A linear 25–400 mM NaCl gradient in buffer B (50 mM sodium phosphate, pH 6.8/2 mM EDTA/5% glycerol/0.02% Nonidet P-40) was used to elute the RT activity. Column fractions were made 50% (vol/vol) with respect to glycerol and stored at −20°C.

RT Assays. Cytoplasmic cell lysates or partially purified proteins were used in RT assays. From 0.01 to 0.3 μg of protein was added to 50-μl reaction mixtures, which contained 50 mM Tris·HCl (pH 8.2), 20 mM dithiothreitol, 50 mM NaCl, 7 mM $MgCl_2$, 0.5 μg of $(rC)_{500}$·$(dG)_{12-18}$, 10 μM dGTP, and 0.01 μM [α-^{32}P]dGTP (800 Ci/mmol; 1 Ci = 37 GBq; Amersham). Reactions were carried out at 37°C for 20 min such that substrates were not limiting, and 5 μl from each reaction was spotted onto Whatman DE81 filters. The filters were washed three times for 5 min each in 2× SSC and once for 5 min in 95% EtOH and dried at room temperature. Filters were analyzed for incorporated [^{32}P]dGTP by liquid scintillation counting; data are presented as units per milligram of protein, where 1 unit of RT is defined as the amount of protein required to incorporate 1 nmol of dGTP into polynucleotide in 20 min at 37°C using poly(rC)·oligo(dG) as the template/primer. All reactions were done in triplicate.

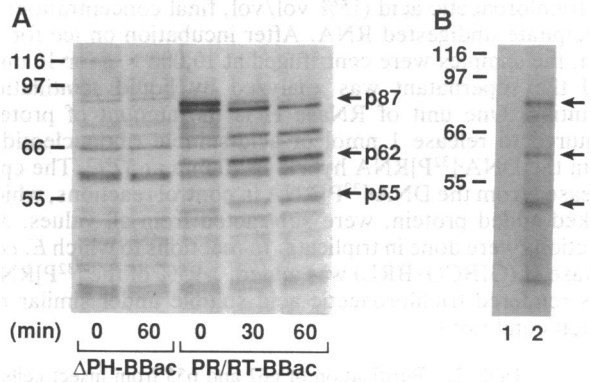

FIG. 2. (*A*) Protein processing following pulse labeling of ΔPH-BBac- or PR/RT-BBac-infected insect cells. After the respective chase time, which is indicated below the lane, the cells were lysed, and released proteins were subjected to SDS/PAGE and exposed to x-ray film. (*B*) Proteins synthesized in *in vitro* translation reactions with either no added RNA (lane 1) or with the 2.8-kb RNA transcribed from pBS-PR/RT (lane 2). Proteins were separated by SDS/PAGE and exposed to x-ray film. Arrows indicate the approximate positions corresponding to p87, p62, and p55.

Table 1. RT and DNA polymerase activities in cell lysates

Lysate	Conditions*	RT specific activity, units/mg	DNA polymerase specific activity, units/mg
ΔPH-BBac	Complete	0.4	3
PR/RT-BBac	Complete	200	550
	− Mg^{2+}	<1	<1
	− Primer	<1	<1
	− Template/primer	<1	<1
	+ Proteinase K	<1	<1
	+ Heat	<1	<1

*Complete, reaction conditions as described in *Materials and Methods*; − Mg^{2+}, no Mg^{2+} was added to the reaction; − Primer, only the respective template was added at 0.5 μg/50-μl reaction mixture; − Template/primer, no template or primer was added to the reaction; + Proteinase K, lysate was pretreated with 0.02 unit of proteinase K per μg of lysate at 37°C for 10 min; + Heat, lysate pretreated for 5 min at 50°C.

DNA Polymerase Assays. The reaction mixture, conditions, and controls used in the RT assay were used to assay the DNA polymerase, except that $(dC)_{350}$·$(dG)_{12-18}$ (Amersham) replaced poly(rC)·oligo(dG) as the template/primer and aphidicolin was included at 200 μM. One unit of DNA polymerase is defined as the amount of protein required to incorporate 1 nmol of dGTP into polynucleotide in 20 min at 37°C using poly(dC)·oligo(dG) as the template/primer. All reactions were done in triplicate.

RNase H Assays. Fractions collected from the Mono S column were used to initiate RNase H assays. A ^{32}P-labeled DNA·RNA hybrid was prepared as described (18, 19). Briefly, 3 μg of M13 ssDNA (13) was added to a reaction mixture containing 15 units of *E. coli* RNA polymerase in the recommended buffer (Promega), 500 μM rNTP (A,C,G), 250 μM UTP, and 0.05 μM [α-^{32}P]UTP (3000 Ci/mmol; Amersham). After 40 min at 37°C, the reaction was stopped by incubation at 75°C for 10 min. The DNA·[^{32}P]RNA hybrid was purified on a Sephadex G-50 (Pharmacia) column and used for RNase H assays in a buffer containing 50 mM Tris·HCl (pH 8.2), 20 mM dithiothreitol, 70 mM NaCl, and 7 mM $MgCl_2$. Between 0.01 and 0.5 μg of protein was added to the RNase H reactions, which were carried out at 37°C for 20 min such that the substrates were not limiting. At the end of the reaction, yeast tRNA was added to 400 μg/ml, followed by trichloroacetic acid (15% vol/vol, final concentration) to precipitate undigested RNA. After incubation on ice for 15 min, the samples were centrifuged at 10,000 × *g* for 10 min, and the supernatant was analyzed by liquid scintillation counting. One unit of RNase H is the amount of protein required to release 1 nmol of acid-soluble ribonucleotides from the DNA·[^{32}P]RNA hybrid in 20 min at 37°C. The cpm released from the DNA·[^{32}P]RNA in control reactions, which lacked added protein, were subtracted from all values. All reactions were done in triplicate. In reactions to which *E. coli* RNase H (GIBCO/BRL) was added, >95% of the [^{32}P]RNA was rendered trichloroacetic acid soluble under similar reaction conditions.

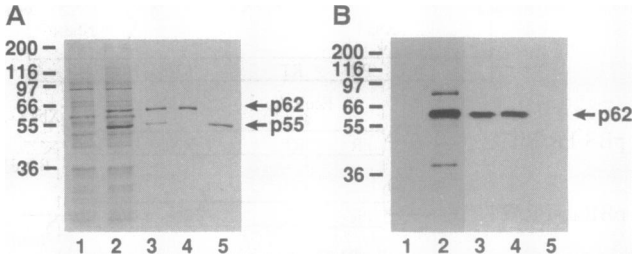

FIG. 3. Analysis of proteins by SDS/PAGE and staining with Coomassie brilliant blue (*A*) or Western blot analysis (*B*). (*A*) Lane 1, proteins from ΔPH-BBac-infected cell lysates; lane 2, proteins from PR/RT-BBac-infected cell lysates; lane 3, p62, and p55 co-eluted from ssDNA column; lane 4, p62 eluted from Mono S column; lane 5, p55 eluted from Mono S column. (*B*) Lanes 1–5 are the same as lanes 1–5 in *A*; blotted proteins were incubated with anti-ΔPR antiserum as described in *Materials and Methods*.

Amino-Terminal Sequence Analysis. Proteins were separated by SDS/PAGE, electroblotted to a poly(vinylidene difluoride) membrane (Bio-Rad), and stained with Coomassie brilliant blue R-250 according to the manufacturer's instructions. The isolated proteins were subjected to amino-terminal sequence analysis on a Porton model 2090 sequencer (Beckman).

Western Blot Analysis. Lysates from baculovirus-infected TN5 cells and proteins purified from the lysates were subjected to electrophoresis in SDS/PAGE and transferred to a nitrocellulose membrane. The protein blot was decorated with the anti-ΔPR antiserum at a 1:10,000 dilution using the ECL Western blot system (Amersham).

RESULTS

Expression of the PR and RT Domains of RTBV. To determine the function(s) of proteins encoded by ORF 3 of RTBV, which was previously demonstrated to encode the capsid protein and predicted to encode a PR and RT, we attempted to express the proteins in *E. coli*. In initial experiments, the PR/RT sequence represented in pBS-PR/RT (Fig. 1) was cloned into the expression plasmid pT7-7 (12). While the plasmid was retained in transformed cells, there was no accumulation of the expected protein(s). When a similar approach was used to express a portion of the predicted RT domain (plasmid pT7-ΔPR; Fig. 1) a 57-kDa protein that included the carboxyl-terminal portion of the RT domain accumulated to significant levels (G.S.L., unpublished data). The 57-kDa protein was subsequently isolated and used to produce antibodies in rabbits. Due to the difficulties in expressing the PR/RT domain in *E. coli*, we turned to the use of baculovirus expression vectors and insect cell cultures to express the PR/RT portion of the RTBV ORF 3.

TN5 insect cells were infected with either a recombinant baculovirus that contained no RTBV sequences (ΔPH-BBac) or PR/RT-BBac, a recombinant baculovirus derived from pBBac-PR/RT (Fig. 1), which contains the portion of ORF 3 predicted to encode the PR and RT activities. At 44 hr postinfection, the TN5 insect cells were incubated in medium containing [^{35}S]methionine for 15 min, then washed exten-

Table 2. Purification of p62 and p55 from insect cells infected with PR/RT-BBac

Purification step	Species	Protein, mg	RT		DNA polymerase	
			Activity, units	Specific activity, units/mg	Activity, units	Specific activity, units/mg
Cytoplasmic lysate	p62/p55	28.0	5600	200	15,400	550
ssDNA-cellulose	p62/p55	1.6	850	530	3,000	1800
Mono S	p62	0.2	420	2100	900	4500
Mono S	p55	0.3	270	900	1,400	4600

sively with PBS, followed by incubation for 0–60 min in medium containing unlabeled methionine (see *Materials and Methods*). Immediately after labeling, an 87-kDa polyprotein was specifically present in PR/RT-BBac cell lysates and is the primary translation product of the PR/RT ORF (Fig. 2*A*). The 87-kDa polyprotein was apparently processed, with the concomitant accumulation of a 62-kDa protein followed closely by the appearance of a 55-kDa protein, designated p87, p62, and p55, respectively. To determine whether the processing of p87 was due to the RTBV PR or an insect cell PR(s), the PR/RT domain was transcribed *in vitro* from pBS-PR/RT and translated in reticulocyte lysates containing [^{35}S]methionine (Figs. 1 and 2*B*). Since in the *in vitro* reactions the labeled protein band pattern is nearly identical to the *in vivo*-labeled PR/RT ORF-derived proteins, we conclude that the PR encoded by the PR/RT ORF is most likely responsible for the processing of the p87 polyprotein.

Activities of the PR/RT Polyprotein Expressed in Insect Cells. To determine whether or not ORF 3 encodes the enzymes necessary for the replication of RTBV as predicted (2), cell lysates were assayed for RT and DNA polymerase activities. As shown in Table 1, cytoplasmic lysates prepared from cells infected with PR/RT-BBac contained a RT activity that was 500-fold greater than such activity in lysates of ΔPH-BBac-infected cells. Control reactions that contained added protein but did not contain either Mg^{2+}, primer, or template/primer exhibited negligible RT activity (Table 1). $MnCl_2$ at 0.7 mM could be substituted for 7 mM $MgCl_2$, with a corresponding 30% reduction in RT activity (G.S.L., unpublished data). DNA polymerase activity was assayed in the same lysates using similar reaction conditions except that the added template/primer was poly(dC)·oligo(dG). Aphidicolin was added to specifically inhibit the insect α DNA polymerase and baculovirus DNA polymerase in these lysates (17, 20). DNA polymerase activity was ≈180-fold greater in PR/RT-BBac-infected TN5 cell lysates than in ΔPH-BBac-infected cell lysates and was dependent on added Mg^{2+}, primer, and template (Table 1).

Purification of the p62 and p55 Proteins. The p62 and p55 proteins were purified from lysates of PR/RT-BBac-infected cells using a ssDNA-cellulose column followed by a Mono S column (see *Materials and Methods*). While p62 and p55 coeluted from the ssDNA-cellulose column, they were eluted in separate fractions from the Mono S column (Fig. 3*A*). Both the purified p62 and p55, as determined by SDS/PAGE and staining of proteins with Coomassie blue, yielded a single homogenous protein band and were estimated to be >90% pure. p62 and p55 contain both RT and DNA polymerase activities (Table 2). The specific activities of p62 and p55 at each step in the purification process are given in Table 2. While p62 is ≈2.5 times more active than p55 in the RT assay, the proteins have equivalent DNA polymerase activities on a molar basis.

Mono S column fractions that contained the p62 and p55 proteins were also used to initiate RNase H assays. The fractions containing p62 did not contain detectable RNase H activity, whereas the fractions that contained p55 had RNase H activity proportional to the concentration of p55 protein (Table 3). This activity was dependent on Mg^{2+}. Neither fraction contained detectable RNase A-type activity (G.S.L., unpublished data).

Table 3. RNase H activity of p62 and p55

Species	Divalent cation	RNase H specific activity, units/mg
p62	Mg^{2+}	<1
p55	Mg^{2+}	200
p55	None	<1

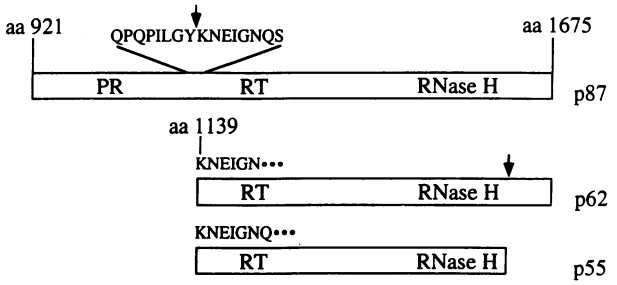

FIG. 4. Summary of processing of the p87 polyprotein encoded by the PR/RT ORF (see Fig. 1). The numbering of the amino acids corresponds to that used for ORF 3 of RTBV (2). Arrows mark processing sites. Amino-terminal sequences determined for p62 and p55 are indicated using the single-letter amino acid code and are aligned with the corresponding amino acid sequences predicted for the p87 polyprotein (2). Dots indicate continuity of the adjacent amino acids with the respective polypeptide.

Amino-Terminal Sequence Analysis of p62 and p55. The purified p62 and p55 proteins were subjected to automated amino-terminal amino acid sequence analysis, which revealed that the amino-terminal sequence of both proteins is identical to the deduced sequence of a translation product of ORF 3 beginning at amino acid 1139 (Fig. 4 and ref. 2). The calculated molecular mass of a protein starting at amino acid 1139 and terminating at the stop codon predicted for ORF 3 is 62,129. On the basis of these data, it is predicted that a polypeptide of ≈7 kDa is cleaved from the carboxyl terminus of p62, resulting in p55 (Fig. 4).

Antibody Recognition of p62. Antiserum from rabbits immunized with the ΔPR protein was used to probe nitrocellulose-bound proteins from PR/RT-BBac-infected cytoplasmic cell lysates and the purified p62 and p55. The anti-ΔPR antiserum recognizes p62 as well as higher and lower M_r proteins in the PR/RT-BBac-infected cell lysates but does not recognize p55 (Fig. 3*B*). This suggests that the anti-ΔPR antiserum recognizes the carboxyl-terminal domain of p62, which is not present in p55 (Fig. 4).

DISCUSSION

In the present study, we demonstrate that the enzymatic activities of proteins derived from ORF 3 of RTBV include a putative PR, RT, DNA polymerase, and RNase H. The RTBV-derived p87 polyprotein that was produced in insect cells was processed, most likely by the PR in p87, to yield two proteins of apparent masses of 62,000 and 55,000 Da (p62 and p55). The conclusion that p87 is processed by the RTBV PR is supported by the results from pulse-labeling experiments in insect cells and *in vitro* studies (Fig. 2), though this does not exclude the possible involvement of a PR present in both insect cells and reticulocyte lysates. The difference in molecular mass between p62 and p55 is apparently due to additional proteolytic processing of p62, which removes ≈7 kDa from the carboxyl terminus, resulting in p55.

On a molar basis, p62 and p55 exhibit equivalent levels of DNA polymerase activity; however, p62 is ≈2.5 times more active as a RT than is p55 (Table 2). The RNase H domain is inactive in p62, whereas it is active in p55 (Table 3). Taken together, these results suggest that carboxyl-terminal processing of p62 is required to activate the RNase H domain but do not exclude the possibility that p55 is also modified in some other way—for example, by phosphorylation. It is interesting to note that the inactive/active state of the RNase H domain apparently has an effect on the RT but not the DNA polymerase activity. A p62 that lacks RNase H activity but has high RT activity may be important in reverse transcribing the terminally redundant genome-length viral transcript. The

activation of an RNase H domain in a RT by proteolytic processing or other modifications has not been described previously. The *in vivo* significance of this finding, however, remains to be determined.

Due to the lack of evidence for the processing of the CaMV PR/RT polyprotein to release an active RT, the results we have described for processing of the RTBV RT cannot be compared directly to other plant pararetroviruses, of which CaMV is the best studied. Hepatitis B, a mammalian pararetrovirus, produces the viral reverse transcriptase *de novo* (21) and therefore cannot be compared to plant pararetroviruses. However, RTBV shares some similarity in processing of the RT with human immunodeficiency virus (HIV). The HIV gag/pol polyprotein contains the PR/RT/integrase, which is cleaved by the PR to yield p66; p66 has both RT and RNase H activities. p66 forms a homodimer in which one subunit is processed a second time, resulting in a p66/p51 heterodimer (22, 23). The processing step removes the carboxyl-terminal 15-kDa RNase H domain from p66, resulting in p51 that lacks both RNase H activity and significant RT activity (24). It is thought that p51 performs a nonenzymatic role in the heterodimer (25).

The processing of the RTBV PR/RT polyprotein superficially appears to follow a similar mechanism as that of the HIV polyprotein, although with different end results; p62 lacks RNase H activity, whereas p55 contains RNase H as well as RT and DNA polymerase activities. Western blot analysis of the purified p55 determined that it was free of detectable p62, demonstrating that p55 is independent of p62 for activity and that a p62/p55 heterodimer, if formed, is not stable during purification. This contrasts with the HIV heterodimer, which is stable under comparable purification conditions (26).

Collectively, these findings allow us to better understand the RTBV RT in terms of the *in vitro* activities that are associated with p62 as well as in the activation of the RNase H domain in p55 and may reflect the enzymatic requirements necessary for a plant pararetrovirus RT to complete the viral replication cycle.

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