Proteolytic Processing of *pol-TYB* Proteins from the Yeast Retrotransposon Ty1

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Using antibodies directed against the *TYB1* protein of the transpositionally competent retrotransposon Ty1-H3, we have identified three mature proteins of 23, 60, and 90 kDa and processing intermediates of 140 and 160 kDa that are derived from the 190-kDa *TYA1-TYB1* polyprotein. Mature proteins and variable amounts of the precursors cofractionate with Ty viruslike particles. The map locations and precursor-product relationships of mature *TYB1* polypeptides suggest that p23 is Ty1 protease, p90 is integrase, and p60 contains reverse transcriptase and RNase H. Immunoprecipitation and immunoblot analyses of Ty1 proteins show that p190 is cleaved to form p160. The p160 intermediate is cleaved to form p23 and p140, and p140 is cleaved to form p90 and p60. Processing of *TYB1* proteins is dependent on Ty1 protease. Immunoblot analysis of *TYB* proteins from different Ty1 isolates reveal that correct processing of *TYB1* proteins is a characteristic of functional Ty1 elements, whereas aberrant processing is a common defect found in transposition-incompetent elements.

The *Saccharomyces cerevisiae* Ty1, Ty2, Ty3, and Ty4 elements belong to a widely distributed group of mobile genetic elements called retrotransposons (4, 7). These elements are similar to retroviral proviruses but do not appear to be infectious. For Ty1 elements, it has been shown that transposition proceeds through an RNA intermediate and resembles several steps in the retroviral life cycle (6). During the transposition process, intracellular Ty1 viruslike particles (VLPs) mature and collect in the cytoplasm (22, 40). The cofractionation of Ty1 VLPs, Ty1 proteins, and nucleic acids, a reverse transcriptase activity capable of synthesizing Ty1 DNA, and an integration activity that catalyzes Ty1 transposition events in vitro strongly suggest that the Ty1 VLP is an essential transposition intermediate (19, 22).

An effective way to study Ty transposition is to use a series of recombinant plasmids called pGTy plasmids (6). These consist of the regulated yeast *GAL1* promoter fused to a Ty element at its transcription initiation site and cloned in a high-copy-number 2 μ m shuttle plasmid. A Ty1 or Ty2 element can be marked with selectable genes such as a bacterial gene for neomycin resistance (*neo*) and the yeast *HIS3* gene (9, 15, 23). In the presence of galactose, transposition of marked pGTy elements and chromosomal Ty elements increases to high levels (6).

There are several host genes that are important for Ty transposition. One such gene is *SPT3*, which affects the length and abundance of Ty transcripts (57). *SPT3* mutants have a small amount of a truncated Ty proximately 800 nucleotides at the 5' end. This mutation blocks transposition of endogenous elements by destroying the ability of Ty RNA to act as an mRNA and as a template for reverse transcription (8, 60). However, the *SPT3* gene is not required for transposition of *GAL1*-promoted Ty elements (8).

All Ty elements contain two overlapping genes: *TYA*, which corresponds to *gag* and specifies a protein that has homology to DNA-binding proteins, and *TYB*, which is equivalent to *pol* and specifies a protein that has limited

homology to retroviral protease (PR), integrase (IN), reverse transcriptase (RT), and RNase H (RH) (12, 18, 21, 25, 33, 38, 43, 52, 54, 58). The order of the *pol* domains in the *Drosophila* copia-Ty1 and Ty2 element family is PR-IN-RT-RH, while the order in retroviruses and the *Drosophila* gypsy-Ty3 element family is PR-RT-RH-IN (58). The gene product of *TYB1* is synthesized as a *TYA1-TYB1* fusion protein resulting from a +1 frameshift near the end of *TYA1* that puts *TYB1* in frame with *TYA1* (3, 11, 12, 39, 56). Mutations have been made in all *TYB1* homology domains. These mutant elements are transposition defective and manifest characteristic biochemical and morphological phenotypes (1, 19, 22, 44, 60, 61).

TYA1 and *TYB1* proteins are processed by Ty1 PR in the maturing particle (1, 44, 60). For Ty1 elements, proteolytically processed *TYA1* proteins form the structural core of Ty1 VLPs. Protease-defective Ty1 elements synthesize immature VLPs containing unprocessed *TYA1* and *TYA1-TYB1* proteins. The precursor-product relationships of the *TYA1* proteins (1, 44, 60) and the identification of a putative Ty1 IN polypeptide have been described previously (19, 22). We are interested in Ty PR because of its possible role in regulating Ty transposition (15) and its striking similarities to retroviral PR (1, 18, 38, 43, 44, 60). In this report, we have identified and mapped the polypeptides that correspond to Ty1 PR, Ty1 IN, and Ty1 RT/RH and determined the relationship of *TYB1* proteins cleaved from the *TYA1-TYB1* polyprotein. This analysis has allowed us to construct a processing pathway for *TYB1* proteins.

MATERIALS AND METHODS

Yeast strains and media. All plasmids were in strains BWG1-7A (*MATa adel-100 ura3-52 leu2-3 leu2-112 his4-519 GAL*), GRF167 (*MATa his3- Δ 200 ura3-167 GAL*), or JSS56-11B (*MATa his3- Δ 1 leu2-3 leu2-112 trp1-289 ura3 GAL*), or their isogenic *spt3-101* derivatives, and are listed in Table 1. All yeast media were those described by Sherman et al. (49).

Nucleic acid manipulations. Various segments of *TYB1* from Ty1-H3 (Fig. 1; Table 2) were subcloned into the

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TABLE 1. Yeast strains

Strain	Genetic background	Plasmid ^a	Source or reference
DG397	BWG1-7A	pGTy1-H3 ^{neo}	15
DG485	BWG1-7A	pGTy1-H1 ^{neo}	15
DG490	BWG1-7A	pGTy1-H4 ^{neo}	This study
DG496	BWG1-7A	pGTy1-588 ^{neo}	15
DG586	GRF167	pGTy1-H3HIS3	This study
DG681 ^b	DG531	pCGS286 ^c	60
DG919	GRF167	pGTy1-H3HIS3(<i>tyb-1702</i>) ^d	This study
DG937 ^b	DG789	pCGS286 ^c	This study
GRY458	JSS56-11B	pGTy1-H3HIS3HH4B#84	55
JB160	GRF167	pCGS329 ^e	6
JB314	BWG1-7A	pCGS286 ^c	6

^a All plasmids are high-copy-number 2 μ m-shuttle vectors containing the yeast *URA3* gene except pGTy1-H3HIS3HH4B#84, which contains a *TRP1* gene in place of *URA3* (55).

^b Strains DG681 and DG937 are isogenic derivatives of strains BWG1-7A and GRF167, respectively, which contain the *spt3-101* mutation (57).

^c pGAL1/*lacZ*.

^d Mutant defective in Ty1 PR (60).

^e pGAL1 vector.

appropriate pATH-*trpE* expression vector (kindly provided by A. Tzagoloff) by standard techniques (17, 37). The genetically marked plasmid pGTy1-H4^{neo} was derived from plasmid pH4, which contains a transposition of Ty1-H4 into the 5' noncoding region of *his3-Δ4* (14). Plasmids were introduced into yeast by the method of Ito et al. (32).

Preparation of bacterial and yeast cell extracts. Protein extracts were made from *Escherichia coli* DH5 carrying *trpE-TYB1* expression plasmids after the bacteria were grown, starved for tryptophan, and induced with 3,β-indoleacrylic acid (Sigma) as described previously (17). Yeast protein extracts were prepared from galactose-grown cultures as described by Youngren et al. (60). Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and visualized by staining with Coomassie blue. Protein concentrations were determined by the method of Bradford (10), using commercially available reagents (Bio-Rad).

Peptide synthesis. Peptides 356, 357, 358, and 359 are derived from the N-terminal region of the Ty1-H3 *TYB* gene that brackets the PR homology domain (18, 38, 43, 50, 52). Peptide 356 contains the amino acids encoded by nucleotides 1792 to 1827, GDLQHFHQDNTK; peptide 357 contains amino acids encoded by nucleotides 1642 to 1677, SDDELP GHLLLD; peptide 358 contains amino acids encoded by nucleotides 1720 to 1755, HSASSNPDIINVV; peptide 359 contains amino acids encoded by nucleotides 1603 to 1638, QKLTESTVNHTN (Fig. 1). Peptide 419 is derived from the RH homology domain of Ty1-H3 (18) and contains amino acids encoded by nucleotides 5425 to 5460, MRLRDGVS GNNL. A noncoding cysteine was added to the N termini of peptides 356 and 359 for coupling to carrier protein. The peptides were assembled on solid supports in an Applied Biosystems model 430A synthesizer (41). Peptides were purified by high-performance liquid chromatography on a reverse-phase C₁₈ column with an increasing acetonitrile gradient (Waters). Aliquots of the purified peptide were analyzed for amino acid content on a Beckman 6300 amino acid analyzer.

Preparation of antisera. Peptides 356 and 359 were coupled (1:1, wt/wt) through a noncoding N-terminal cysteine residue to keyhole limpet hemocyanin, using *m*-maleimidobenzoyl-

N-succinimide ester (Pierce) (27, 36). Peptides 357, 358, and 419 were coupled (1:1, wt/wt) through their N termini to keyhole limpet hemocyanin, using difluorodinitrobenzene essentially as described by Tager (51). For initial immunizations in male New Zealand White rabbits, 1 mg of peptide-keyhole limpet hemocyanin conjugate in Freund's complete adjuvant was used. Subsequent boosts followed at 2-week intervals, using 0.1 mg of the conjugate in Freund's incomplete adjuvant (13). Initial reactivity of the antibodies to their cognate peptides was determined by a two-step enzyme-linked immunosorbent assay using goat anti-rabbit immunoglobulin G-peroxidase and the chromogen 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) (Boehringer Mannheim). To prepare antigen from the fusion proteins, total protein or the insoluble fraction of a bacterial lysate was isolated and subjected to SDS-polyacrylamide gel electrophoresis. The position of the relevant *trpE-TYB1* fusion protein was visualized after precipitation with 4 M sodium acetate or limited staining with Coomassie blue. Antibodies to *trpE-TYB1* fusion proteins were raised in male New Zealand White rabbits by established techniques (27). Antibodies purified from whole sera by affinity chromatography over protein A-Sepharose (Pharmacia) usually resulted in preparations suitable for immunoprecipitation and immunoblotting analyses. Sera were initially tested for cross-reactivity with Ty1 proteins by immunoblotting. *TYB1* antisera recognized *TYB1* precursor proteins isolated from cells expressing a pGTy-PR mutation and mature proteins from cells expressing a wild-type pGTy plasmid. Antisera specific for *TYB1* did not cross-react with Ty proteins isolated from *spt3* mutants (60). Specific cross-reactivity of peptide 359 and 419 antibodies for Ty1 VLP proteins was checked by preincubating immune sera with a 300- to 3,000-fold molar excess of the peptides followed by immunoblotting. The Ty1 VLP and B2 antisera have been described previously (1, 60).

Isolation of Ty VLPs. The VLPs were isolated as described previously (19) except for the following minor modifications. Strain GRY458 was used to isolate Ty1 VLPs in most experiments because this strain gave a fivefold-higher yield than other strains. It is likely that the higher Ty VLP yield results from increased pGTy plasmid stability. In some preparations, Ty1 VLPs were concentrated by column chromatography using Superose 6A (Pharmacia). Ty VLP purification was monitored by SDS-polyacrylamide gel electrophoresis and reverse transcriptase activity (22).

Immunoblotting. Immunoblots were prepared by the method of Towbin et al. (53). Total yeast proteins and Ty VLPs were separated on SDS-polyacrylamide gels, with acrylamide concentrations ranging from 7.5 to 15%. Initially, proteins were electrophoretically transferred to nitrocellulose, but in most experiments polyvinylidene difluoride membranes (Immobilon-P; Millipore) were used. Filters were treated with 5% (wt/vol) bovine serum albumin (fraction V; Sigma) or 5% (wt/vol) nonfat dry milk dissolved in phosphate-buffered saline (PBS; 0.15 M NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.2) or NET buffer (50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 5 mM Na₂EDTA, 0.25% [wt/vol] gelatin, 0.05% [wt/vol] Triton X-100) for several hours at room temperature to block nonspecific binding of antisera. Antibodies were added in about 10-fold excess, as determined by titration experiments, and the filters were incubated for 2 to 16 h at room temperature. The filters were washed in blocking buffer containing 0.05% (vol/vol) Nonidet P-40 (Sigma) and 0.01% (wt/vol) SDS with several changes and then soaked in blocking buffer minus detergents. Antibody-antigen complexes were detected with

¹²⁵I-labeled protein A (33 mCi/mg; Amersham) diluted to a final concentration of 1 µCi/ml in blocking buffer and then subjected to autoradiography. Unbound protein A was removed by multiple washes in PBS plus detergents. The apparent sizes of relevant proteins were determined by electrophoresis alongside prestained protein markers (Amersham). Proteins transferred to Immobilon-P membranes were visualized by staining with colloidal gold as described by the supplier (Enprotech). The protein markers were myosin H chain (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa), aprotinin (6.5 kDa), insulin b chain (3.4 kDa), and insulin a chain (2.35 kDa).

Radioactive labeling of cells and immunoprecipitation. To induce high levels of Ty transposition, strains were precultured in SC-uracil medium containing 2% (wt/vol) glucose at 30°C, diluted 15-fold into yeast nitrogen base minimal medium supplemented with histidine and 2% (wt/vol) galactose (Sigma or Pfanstiehl) (YNB+His+Gal), and grown overnight at 20°C. For radioactive labeling, the optical density at 600 nm of the cells was adjusted to 2 with fresh YNB+His+Gal, either [³H]leucine (150 Ci/mmol; Amersham) or [³⁵S]methionine (800 Ci/mmol; Amersham) was added to a final concentration of 500 or 200 µCi/ml, respectively, and the cells were incubated at 20°C. For pulse-chase experiments, cells were pulse-labeled with [³⁵S]methionine or [³H]leucine for 30 min and then chased with a 1,000-fold excess of unlabeled methionine or leucine (Sigma) for different time periods. Immunoprecipitation experiments were carried out with fixed *Staphylococcus aureus* cells (IgG Sorb; The Enzyme Center) essentially as described by Schauer et al. (47). The IgG Sorb was prepared according to the supplier's recommendations. Immunoprecipitates suspended in sample buffer were stored at -20°C or immediately subjected to SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide slab gels (12 cm by 14 cm by 1 mm; Hoefer) were prepared by the method of Laemmli (34). These gels were usually subjected to electrophoresis for 16 h at 10 mA. Mini-SDS-polyacrylamide slab gels from Enprotech or Novex were used according to the suppliers' specifications. Typically, the minigels were run for 90 min at 30 mA. For autoradiography, the gels were fixed for 20 min in 50% (vol/vol) methanol-10% (vol/vol) acetic acid and then soaked in Amplify enhancing solution (Amersham) for 30 min with gentle rocking. The gels were dried under vacuum before exposure to XAR X-Omat film (Eastman Kodak). Molecular weight estimates of the relevant proteins were made as described above.

RESULTS

Detection of *TYB1* proteins by using antisera raised against *trpE-TYB1* fusion proteins or synthetic peptides. We continued our studies on the genetic organization of the Ty1-H3 *TYB1* gene (60) by defining the map positions and processing cascade of *TYB1* proteins. To obtain large amounts of *TYB1* protein for immunization, we expressed portions of the *TYB1* gene as *trpE* fusion proteins in *E. coli*, using pATH expression plasmids (Table 2; Fig. 1; Materials and Methods). Fusion proteins isolated by preparative gel electrophoresis were used to immunize rabbits, and immune sera were collected for further characterization.

As an example, we present the steps in developing antiserum B3 (Fig. 2). Total proteins extracted from strain DH5 containing the expression plasmid pB3 (Fig. 2A, lane 1), the

TABLE 2. *trpE-TYB1* expression plasmids

Fusion protein	Restriction enzyme sites at termini of inserted Ty1-H3 DNA	Ty1-H3 nucleotide no. ^a	pATH vector	Size of fusion protein (kDa)
B1	<i>EcoRV-EcoRV</i>	1900-2779	2	70
B2	<i>EcoRV-PvuII</i>	2779-3944	2	80
B3	<i>BglII-SalI</i>	1702-2173	11	55
B5	<i>PvuII-HindIII</i>	3944-4627	11	62
B7	<i>BglII-HindIII</i>	3301-4627	11	86
B8	<i>HindIII-BglIII</i>	4627-5561	3	72

^a As reported by Boeke et al. (5).

pATH11 vector alone (lane 2), or a heterologous plasmid (lane 3; pGRMAT was kindly provided by J. Strathern) were separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. Strain DH5(pB3) produced an abundant 55-kDa fusion protein, whereas cells containing pATH11 produced a 37-kDa *trpE* protein. Immune sera raised against the fusion proteins were initially tested by immunoblotting using total yeast protein isolated from cells expressing pGTy1-H3 or a pGTy1-H3(*tyb-1702*) PR mutation, purified Ty1 VLPs, or a mock Ty VLP fraction from an isogenic *spt3* mutant, DG681, that produces undetectable levels of Ty1 proteins (60). Antiserum B3 was incubated with an Immobilon-P membrane (Fig. 2B) containing a mock Ty VLP fraction from strain DG681 (lane 1) and wild-type Ty1 VLPs (lane 2). The Ty VLP-dependent appearance of p90-*TYB1* as well as three putative precursors was evident after treatment of the filter with ¹²⁵I-protein A.

To identify proteins from the ends of *TYB1*, antisera were generated to synthetic peptides that are located near the frameshift region or C terminus (Materials and Methods; Fig. 1). Of the four peptides located near the *TYA1-TYB1*

FIG. 1. Map position of Ty1-H3 gene fusions and peptides used for immunization. At the top is the structure of Ty1-H3. The DNA is represented by rectangles containing the *TYA* and *TYB* genes; the triangles are Ty long terminal repeats. The two boxes below the Ty element represent the coding sequences of *TYA* and *TYB*. *TYA* is analogous to the retroviral *gag* gene; *TYB* is analogous to the retroviral *pol* gene. A vertical line denotes the frameshift region separating *TYA* and *TYB*. Four domains of *TYB-pol* have been identified by sequence homologies: PR, IN, RT, and RH. A region of *TYB* that is quite divergent and shows no homology with retroviral proteins is located between IN and RT and is covered by the B2 fusion protein. Antisera were raised in rabbits against synthetic peptides 359 and 419 (see Materials and Methods for map positions and sequences of the peptides) or *trpE-TYB* fusion proteins (shaded boxes; B1, B2, B3, B5, B7, and B8). Table 1 shows the map positions of the fusion proteins (corresponding nucleotides would proceed from left to right in this figure).

FIG. 2. Analysis of B3 antiserum. (A) SDS-polyacrylamide gel of total protein isolated from *E. coli* DH5 carrying the *trpE-TYB1* fusion plasmid pB3 (lane 1), the *trpE* vector pATH11 (lane 2), or a heterologous plasmid pGRMAT (lane 3). The proteins were analyzed on a 10% gel and then stained with Coomassie blue. Positions of the 55-kDa B3 fusion protein and the 37-kDa *trpE* protein are indicated. (B) Immunoblot analysis with antiserum B3. Ty VLP isolations from a *spt3* mutant DG681 (lanes 1 and 3) or wild-type strain DG397 (lanes 2 and 4) and the resulting protein samples (20 µg per lane) were subjected to electrophoresis in an SDS-12.5% polyacrylamide gel. The proteins were electroblotted to an Immobilon-P membrane and incubated with antiserum B3. Immune complexes were detected by using 125 I-protein A and autoradiography (lanes 1 and 2). The same membrane was then stained for protein with colloidal gold (lanes 3 and 4). The estimated sizes for the *TYB1* precursor proteins (190, 160, and 140 kDa) and the mature *TYB1* IN protein (90 kDa) are shown. Also shown is nonspecific cross-reaction observed between antiserum B3 and the p58^{TYA1} precursor (*).

frameshift that were used for immunization, only peptide 359 gave rise to antibodies that adequately cross-reacted with Ty1 proteins. Antisera to peptides 356, 357, and 358 weakly cross-reacted with Ty precursor proteins but did not cross-react with proteins in a size range for Ty1 PR predicted from homology studies (61). Peptide 359 begins at a *TYB1* Gln codon, which is two codons downstream of the *TYA1-TYB1* frameshift (3). The C terminus of peptide 359 is 10 codons upstream from the PR homology domain. Peptide 419 is located between two Asp codons that are conserved in all RH proteins (18) and is 33 codons from the C terminus of *TYB1*.

All of the *TYB1* antisera cross-reacted to various degrees with proteins that are probably derived from *TYA1*, since the cross-reactive proteins comigrated with *TYA1* proteins under all conditions tested (Fig. 2B and 3) (60, 61). The cross-reaction detected by immunoblotting occurred under a variety of blocking and washing conditions.

Cofractionation of *TYB1* proteins with Ty1 VLPs. Ty1-H3 VLPs were isolated from strain GRY458 by differential centrifugation and sucrose gradient sedimentation. The VLP proteins were separated on SDS-polyacrylamide gels and immunoblotted, and the resulting filters were reacted with *TYB1* antisera (Fig. 3). A consistent Ty1 protein pattern was observed with antisera raised against different segments of Ty1 coding sequence. Antiserum 359 cross-reacted with a precursor protein of 190 kDa, a processing intermediate of 160 kDa, and a putative Ty1 PR protein of 23 kDa but not with an intermediate of 140 kDa (Fig. 3). The 190-kDa protein is probably the full-length *TYA1-TYB1* polyprotein because both *TYA1*- and *TYB1*-specific antisera reacted with a precursor of the size (193 kDa) expected from the coding

FIG. 3. Immunoblot analysis of *TYB1* proteins from Ty1-H3 VLPs. Gradient-purified Ty1-H3HIS3 VLPs were isolated from strain GRY458 and separated on SDS-7.5% (B2, B7, B5, B8, and 419), -10% (B3 and B1), or -12.5% (359) polyacrylamide gels. The proteins were transferred to Immobilon-P and screened for cross-reactivity with various *TYB1* antisera (Fig. 1 shows the map positions of the different *TYB1* antisera). Immunoblots were overexposed by increasing the time of autoradiography in order to detect *TYB1* precursors in the VLPs. Cross-reactivity with *TYA1* proteins (*) and Ty1 protein size estimates (in kilodaltons) are indicated. The minor bands observed between p90 and p140 are probably caused by cellular proteolysis because they are present in immunoblots prepared from a Ty PR mutant (61).

potential of Ty1-H3 (Fig. 3 and 4) (1, 44, 60). Antiserum 359 also cross-reacted with *TYA1* proteins, but this cross-reaction was somewhat variable and was not markedly diminished by the addition of excess competitor peptide 359. In

FIG. 4. Immunoprecipitation of *TYB1* proteins from cells expressing pGTy1-H3HIS3. Strain DG586 was induced for Ty transposition by growth in galactose medium, pulse-labeled with [35 S]methionine for 30 min, and chased with excess unlabeled methionine. Samples were taken at 1, 2, 6, and 24 h after addition of unlabeled methionine. Total cell extracts were prepared, and then equal amounts of the labeled proteins were immunoprecipitated with various *TYB1* antisera and analyzed by electrophoresis on a SDS-10% polyacrylamide gel as described in Materials and Methods. The last lane (-Ab) contains a control immunoprecipitation in which antiserum was not added to an exact prepared after a 24-h chase. Molecular size estimates (in kilodaltons) of Ty1 proteins are indicated on the left. The minor bands observed between p90 and p140 are probably caused by cellular proteolysis because they are present in immunoprecipitates from a Ty PR mutant (61).

contrast, addition of excess peptide 359 completely inhibited cross-reaction with p190^{TYA1-TYB1}, p160, and p23 (61).

Antisera B3, B1, B2, and B7 were made against a region of *TYB1* that encodes Ty1 IN (19, 43, 50). These antisera cross-reacted to varying degrees with apparent precursors of 190, 160, and 140 kDa and strongly cross-reacted with a 90-kDa protein that is Ty1 IN (Fig. 3). Fusion proteins B5, B7, B8, and peptide 419 contain the Ty1 RT/RH homology domains (12, 18, 43, 50, 54, 58). The resulting antisera reacted to various degrees with apparent precursors of 190, 160, and 140 kDa and strongly reacted with a 60-kDa protein that is Ty1 RT/RH (Fig. 3). Antiserum to peptide 419 weakly cross-reacted with *TYA1* proteins. However, competition experiments performed with peptide 419 indicate that cross-reaction with *TYA1* protein is not peptide specific (61). To determine whether there might be other *TYB1* proteins that map between p90 and p60, antiserum was raised against the B7 fusion protein because this fusion protein spans the putative p90-p60 cleavage site. Antiserum B7 cross-reacted with both p90 and p60 and the expected set of precursors (Fig. 3). These immunoblots did not reveal any other *TYB1* proteins whose apparent size is greater than 10 kDa that might be located between p90 and p60.

Immunoprecipitation of *TYB1* proteins. Antisera B2, B3, B7, B8, and 359 were used in pulse-chase analysis to determine the precursor-product relationship of *TYB1* polypeptides (Fig. 4). To ensure high levels of *TYB1* protein accumulation, strain DG586 was grown in galactose medium overnight, diluted in fresh inducing medium, pulse-labeled for 30 min with either [³H]leucine or [³⁵S]methionine, and then chased with excess unlabeled amino acid for various time periods. In the earliest chase period of 1 h, p190, p160, and p140 were immunoprecipitated with antisera that recognize Ty1 IN (B2, B3, and B7) and Ty1 RT/RH (B7 and B8). As the chase time increased to 24 h, the mature p90 IN and p60 RT/RH proteins increased in concentration while the labeled precursors gradually decreased in concentration. The levels of p90 and p60 remained unchanged even after a 24- to 36-h chase, suggesting that these mature *TYB1* proteins are stable (61). During the course of these experiments, p160 often appeared as a double band. This may reflect an additional cleavage or modification of p160.

To detect Ty1 PR by immunoprecipitation, we labeled galactose-induced strain DG586 with [³H]leucine or [³⁵S]methionine. [³H]leucine was used because there are no Met codons in the PR domain (12, 18, 38, 52). In addition, immunoblot analysis suggested that Ty1 PR is a relatively small protein of 23 kDa (Fig. 3). We were able to immunoprecipitate p23 PR and the p190 and p160 precursors with antiserum 359 when the cells were labeled with [³H]leucine for several hours (Fig. 5, lane 3). Pulse-chase analysis with antiserum 359 resulted in a pattern of synthesis and turnover of p190 and p160 that was similar to the pattern obtained with other *TYB1* antisera (Fig. 4), but we could not reproducibly detect p23 at every time point (61). This may be a reflection of the low amount of p23 present in galactose-induced cells or the avidity of antiserum 359. As expected, attempts to immunoprecipitate p23 PR with antiserum 359 failed when cells were labeled with [³⁵S]methionine; the only proteins that appeared were the p190 and p160 precursors (61).

Interestingly, p140 did not react with the antiserum 359 by immunoblot (Fig. 3) or immunoprecipitation (Fig. 5, lane 3) analyses. In a parallel experiment, antiserum B3 was able to immunoprecipitate the p140 precursor as well as p90, p160, and p190 (Fig. 5, lane 2). As a control for nonspecific

FIG. 5. Immunoprecipitation of Ty1 PR from cells expressing pGTy1-H3HIS3. Wild-type strain DG586(pGTy1-H3HIS3) and *spt3* mutant strains DG937(pCGS286) were grown in galactose medium, labeled with [³H]leucine for 6 h, immunoprecipitated with antiserum B3 or 359, and analyzed by SDS-polyacrylamide gel electrophoresis on a 4 to 20% gradient gel as described in Materials and Methods. Lanes 1 (DG586) and 4 (DG937) are control immunoprecipitations in which antisera were not added. Lanes 2 (DG586) and 5 (DG937) contain labeled proteins immunoprecipitated by antiserum B3. Lanes 3 (DG586) and 6 (DG937) contain labeled proteins immunoprecipitated by antiserum 359. Longer exposure of the gel did not alter the protein pattern (61). Ty1 protein size estimates (in kilodaltons) are indicated on the left.

reactivity of antisera 359 and B3, immunoprecipitations were performed with an isogenic *spt3* mutant, DG937 (Fig. 5, lanes 5 and 6). These immunoprecipitates did not contain *TYB1* proteins.

Formation of p23 and p60 is dependent on Ty1 PR. We have previously shown that the formation of p90 IN is blocked in strains carrying a marked pGTy plasmid with a mutation in Ty1 PR (60). To determine whether the formation of p23 PR and p60 RT/RH was dependent on Ty1 PR, proteins from wild-type strain DG586 and a Ty1 PR mutant, DG919, were analyzed with antiserum 359 and B8 (Fig. 6A). Mature p23 (antiserum 359, lane 1) and p60 (antiserum B8, lane 1) proteins were immunoprecipitated from strain DG586, but only the p190 precursor was immunoprecipitated from strain DG919 (antisera 359 and B8, lanes 2). Identical results were obtained when Ty VLPs from Ty1 PR mutant strain DG919 were analyzed for processed *TYB1* proteins by immunoblotting (Fig. 6B).

Reactivity of anti-*TYB1* sera with proteins from other Ty1 elements. We determined whether antisera raised against Ty1-H3 *TYB1* IN and RT/RH proteins showed cross-reactivity with proteins from other genetically marked, *GALI*-promoted Ty1 elements (Fig. 7). These elements include two spontaneous Ty1 insertions, Ty1-H1 and Ty1-H4, that are transposition defective, an endogenous chromosomal Ty element Ty1-588 that is transposition competent, as well as the spontaneous transposition insertion Ty1-H3 (6, 14, 15).

Strains carrying genetically marked pGTy1^{neo} (Fig. 7, lanes 1 to 4) or heterologous expression plasmids (lanes 5 and 6) were grown in galactose medium, and total protein was analyzed by immunoblotting using antisera that recognize p90 IN (B2) or p60 RT/RH (B8). Cells with heterologous expression plasmids did not contain immunoreactive Ty1 IN or Ty1 RT/RH protein (Fig. 7, lanes 5 and 6). This result supports the previous observation that yeast cells normally contain very low levels of Ty proteins (22). *GALI*-promoted

FIG. 6. Formation of Ty1 PR and Ty1 RT/RH in cells expressing Ty PR mutant pGTy1-H3HIS3(*tyb-1702*). (A) Wild-type strain DG586 (lane 1) and the Ty PR mutant DG919 (lane 2) were induced for transposition by growth in galactose medium. Cells were labeled with [3 H]leucine for 6 h and immunoprecipitated with antiserum 359 or labeled with [35 S]methionine for 6 h and immunoprecipitated with antiserum B8. Control immunoprecipitations that lack *TYBI* antisera are shown in lane 3. (B) Ty1 VLPs were purified from the Ty PR mutant DG919 and separated by electrophoresis on a SDS-10% polyacrylamide gel. The proteins were transferred to an Immobilon-P membrane and cross-reacted with antisera to Ty1 VLPs, 359, and B8 as described in the Materials and Methods. Ty1-VLP antiserum reacts with *TYAI* proteins (1, 44, 60) and is included so that the size of the p190^{*TYAI-TYBI*} precursor protein could be compared by using antiserum specific to either *TYAI* or *TYBI*. Ty1 protein size estimates (in kilodaltons) are shown on the right.

expression of Ty1-H3*neo* resulted in the synthesis of p90, p60, and variable amounts of immunoreactive *TYBI* precursors (lane 1; strain DG397). The protein pattern obtained from expression of an endogenous transposition-competent Ty1-588*neo* element was similar to that of Ty1-H3 (lane 2; strain DG496). However, the defective Ty1-H1*neo* (lane 3; strain DG485) and Ty1-H4*neo* (lane 4; strain DG490) elements produced altered *TYBI* products. Ty1-H1 contains an unknown mutation that markedly reduces transposition (15). Ty1-H1 failed to synthesize detectable levels of p90 and p60. Instead, this element produced proteins in the size range of p190 and p160 that cross-reacted with antisera B2 and B8. Ty1-H4 contains a deletion that removes some of the PR and IN coding sequence (14) but leaves the reading frame intact, as is evident from the presence of a large *TYBI* protein of approximately 150 kDa that cross-reacted with antisera B2 and B8. The size of this *TYBI* protein is consistent with the size of the deletion in Ty1-H4.

DISCUSSION

An essential step in the life cycle of many retroelements is the formation of VLPs containing *gag*-like and *pol*-like proteins and the formation of retroelement nucleic acid (20). The experiments reported here reveal a retroviruslike precursor-product relationship between the *TYAI-TYBI* polyprotein and mature PR, IN, and RT/RH (Fig. 8). *TYBI* is synthesized as a 190-kDa *TYAI-TYBI* polyprotein that requires a change of reading frame to be expressed (12, 39). The 190-kDa precursor is cleaved by Ty1 PR to form a 160-kDa processing intermediate. The cleavage site required to form p160 may be near the *TYAI-TYBI* frameshift, because all antisera that have been made against *TYAI* proteins do not cross-react with p160 (1, 44, 60, 61). Our

FIG. 7. Characterization of B2 and B8 antisera for cross-reactivity with proteins from different Ty1*neo* elements. Strains DG397 (pGTy1-H3*neo*; lane 1), DG496 (pGTy1-588*neo*; lane 2), DG485 (pGTy1-H1*neo*; lane 3), DG490 (pGTy1-H4*neo*; lane 4), JB160 (pCGS329; lane 5), and JB314 (pCGS286; lane 6) were induced for transposition by growth in galactose medium, and total protein was isolated for immunoblot analysis. Proteins were separated by electrophoresis on an SDS-7.5% polyacrylamide gel, transferred to an Immobilon-P membrane, and cross-reacted with B2 and B8 antisera as described in Materials and Methods.

results suggest that p160 is cleaved to form a p140 processing intermediate and p23, which is probably Ty1 PR. However, it is possible that p160 is not the direct precursor of p23, since the results of pulse-chase immunoprecipitations with peptide 359 antiserum remain equivocal. The p140 intermediate is cleaved by Ty1 PR to form p90, which was previously shown to be Ty1 IN (19), and p60, which our results suggest is Ty1 RT/RH. The proposed *TYAI-TYBI* processing cascade is consistent with the map positions and phenotypes of mutations made in *TYBI* PR, IN, and RT/RH homology domains (1, 5, 19, 22, 44, 60, 61). In addition, the studies reported here will complement ongoing efforts to identify Ty1 PR cleavage sites.

It is interesting that an RT/RH-IN intermediate is conserved in the maturation of *TYBI* and avian and murine retroviral *pol* proteins (16). The presence of RT/RH and IN in a multidomain protein may be evolutionarily and functionally significant. For example, it has been reported that murine leukemia virus RT/RH and IN can be associated as a complex involving a disulfide bond(s) (29). Although the biological relevance of this complex remains to be determined, the association of RT/RH and IN may be enhanced if both proteins derive from a common processing intermediate. Furthermore, the avian retrovirus RT/RH-IN (β) and RT/RH (α) complex is a major *pol* product found in virions

FIG. 8. Scheme for Ty1-H3 *TYB* processing. p190^{TYA-TYB}, the 190-kDa product of the *gag-TYA* and *pol-TYB* genes, is cleaved near the frameshift region (vertical line separating *TYA* and *TYB*). This cleavage releases p160^{TYB}, which is further processed to Ty1 PR (23 kDa) and p140^{TYB}. Cleavage of p140^{TYB} produces Ty1 IN (p90) and Ty1 RT/RH (p60). The dotted lines indicate that p160 and p23 may be encoded by both *TYA* and *TYB*. Also shown is the 58-kDa *TYA* precursor and 54-kDa processed product (1, 44, 60). These proteins are the major structural components of Ty VLPs (1).

and possesses several enzymatic activities, which include RT, DNA-dependent DNA polymerase, RH, and DNA endonuclease (24). Therefore, β can be viewed as a processing intermediate and as a functional RT/RH and possibly IN protein.

To more fully understand the processing pathway of *TYA* and *TYB* proteins, the protein spanning the Ty1 frameshift must be identified (Fig. 8). Belcourt and Farabaugh have recently shown that Ty1 frameshifting occurs at two overlapping Leu codons (3). The antiserum used to detect the 23-kDa Ty1 PR protein was derived from peptide 359, which begins with a *TYB* Gln residue two codons downstream of the frameshift. Therefore, it is possible that p23 is the mature Ty1 protein that spans the frameshift. N-terminal sequence analysis of p23 or the p160 precursor should indicate whether these proteins extend into *TYA* (Fig. 8). A similar situation exists for murine leukemia virus, in which PR is split between the *gag* and *pol* reading frames (59), but not for human immunodeficiency virus type 1, in which PR is wholly *pol* encoded (46).

The Ty1 and Ty2 IN proteins appear to contain two distinct regions. The N-terminal third to one-half of the *TYB* IN protein sequence contains blocks of homology with IN proteins from all Ty elements, other retrotransposons, and several retroviruses (25, 33, 43, 50, 54). Antiserum B1 is directed against this region of *TYB*. Antigenic conservation between Ty1 and Ty2 IN proteins has been shown by cross-reactivity of B1 antiserum with Ty2 IN proteins of 95 and 100 kDa from the transposition-competent Ty2-117 and Ty2-917 elements, respectively (61). The rest of IN, which is present on the B2 fusion protein, is conserved within the Ty1 or Ty2 element families but is quite divergent between families and is not homologous with other IN proteins. Additional immunoblots using antiserum B2 indicate that the divergent region in *TYB* is not conserved in Ty2 elements (61). Taken together, the reactivities of B1 and B2 antisera clearly place both the conserved region shared between various IN proteins and the divergent region within p90^{TYB}. It will be interesting to determine whether these Ty IN proteins require both regions for activity and insertional specificity.

The antibody reagents developed in this study are being used to study factors that regulate Ty transposition. The

yeast genome contains transpositionally competent, transcriptionally active Ty elements, such as Ty1-588, as well as mutant Ty elements (5, 15). In the examples presented here, naturally occurring mutant elements Ty1-H1 and Ty1-H4 are defective in forming mature *TYB* proteins p60 and p90. Instead, these elements produce proteins in the size range of 150 to 200 kDa. Another naturally occurring transposition-defective Ty1 element, Ty1-173, produces a protein of about 97 kDa instead of p90^{TYB} (5). These results suggest that the inability to produce fully mature *TYB* proteins is a common defect associated with mutant elements. This could be caused by defects in Ty1 PR or in the cleavage sites of the *TYB* precursors. It will be interesting to determine whether a similar pattern emerges for Ty2, Ty3, and Ty4 elements. Recently, a transposition-defective Ty3 element has been characterized (25, 26). Ty3-2 produces an IN protein that is somewhat smaller than that produced by the transposition-competent element Ty3-1 because of a single nucleotide deletion 75 codons from the C terminus. Consequently, these mutant Ty elements have retained the ability to synthesize Ty proteins. This is in marked contrast to retroelements of multicellular organisms, many of which are grossly rearranged and contain multiple chain-terminating mutations. These include LINE retrotransposons of mammals, plants, insects, and protozoa (30), 17.6 and gypsy retrotransposons of *Drosophila melanogaster* (31, 35), and VL30 (2), ETn (48), and IAP retrotransposons of rodents (42, 45). Defective Ty elements could be involved in regulating transposition if they contain dominant negative mutations (28). This would also create a selection for maintenance of mutant Ty elements. Further biochemical and genetic analysis of chromosomal Ty elements will be required to understand their role in Ty biology.

In summary, Ty1 elements retain a *pol*-like protein cleavage pathway despite a different *pol*^{TYB} gene organization. Since Ty1 elements belong to a larger group of retrotransposons in which the order of the RT/RH and IN domains is reversed (18, 58), a Ty1-like processing strategy to form mature *pol* proteins will probably be utilized by other members of this group.

ACKNOWLEDGMENTS

This research was sponsored by the National Cancer Institute under contract NO1-CO-74101 with ABL.

We are grateful to S. Showalter (Program Resources, Inc., NCI-Frederick Cancer Research and Development Center, Frederick, Md.), Y. Kim (PRI), and P. Wesdock (ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center) for assistance with peptide synthesis and antisera preparation, T. Burkett, M. J. Curcio, R. Fishel, and G. Sharon for helpful comments on this work, and A. Arthur (ABL-Basic Research Program), R. Frederickson (PRI), and P. Hall (ABL-Basic Research Program) for preparation of the manuscript.

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