Depurination of Brome Mosaic Virus RNA3 in Vivo Results in Translation-dependent Accelerated Degradation of the Viral RNA*

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Pokeweed antiviral protein (PAP) is a ribosome-inactivating protein isolated from the pokeweed plant (Phytolacca americana) that exhibits antiviral activity against several plant and animal viruses. We have shown previously that PAP depurinates Brome mosaic virus (BMV) RNAs in vitro and that prior incubation of these RNAs with PAP reduced their synthesis in barley protoplasts. To investigate the post-transcriptional effect of PAP on viral RNA in vivo, we transcribed BMV RNA3 and expressed PAP in the yeast, Saccharomyces cerevisiae, which is a surrogate host for BMV. With an inducible transcription system, we show that the half-life of RNA3 in PAP-expressing cells was significantly less than in cells expressing PAPx, its enzymatically inactive form. PAP bound to RNA3 and depurinated the RNA within open reading frames 3 and 4 and within untranslated regions of the RNA. The depurinated RNA was associated with polysomes, caused ribosomes to stall at the point of depurination, and was targeted for accelerated degradation by components of the No-go decay pathway. As a consequence of translation elongation arrest and increased RNA degradation, expression of PAP in yeast also decreased the level of protein 3a, encoded by the 5’-proximal open reading frame 3 of BMV RNA3. These data provide the first evidence of viral RNA depurination in vivo by any ribosome-inactivating protein and support our hypothesis that depurination contributes to the antiviral activity of PAP, by enhancing viral RNA degradation and reducing translation of viral protein product.

Pokeweed antiviral protein (PAP)³ is a 29-kDa ribosome-inactivating protein isolated from the leaves of the pokeweed plant, Phytolacca americana (1, 2). Like all ribosome-inactivating proteins, PAP is an N-glycosidase that cleaves an adenine from the highly conserved sarcin/ricin loop within domain VI of the large rRNA (3, 4). This depurination disrupts the binding ability of elongation factor eEF-2; hence, protein synthesis is inhibited at the translocation step (5).

In addition to affecting translation, PAP also has broad-range antiviral activity against several animal and plant viruses (6–8). The antiviral property was initially hypothesized to be due to cytotoxic effects caused by reduction in cellular translation. However, subsequent characterization of PAP mutants uncovered variants that maintained antiviral activity without rRNA depurination and consequent inhibition of translation (9, 10). Studies have shown that, in addition to depurination of RNA, PAP depurinates Brome mosaic virus (BMV) RNAs (11) and human immunodeficiency virus, type 1 RNA in vitro (12). Moreover, pretreatment of BMV RNAs with PAP resulted in decreased synthesis of these viral RNAs in barley protoplasts (13). These data suggest that, in addition to depurinating RNA, PAP may also target viral RNAs in vivo; however, this possibility has not yet been demonstrated.

BMV is a positive-sense, single-stranded RNA virus that possesses a tripartite genome, of which two RNAs are monocistronic (RNAs 1 and 2) and the third RNA is dicistronic (RNA3) (14, 15). The 5’-ends of all three RNAs are capped with 7-methylguanylate and the 3’-ends contain a conserved tRNA-like structure without a poly(A) tail (16, 17). RNA1 encodes replication protein 1a, with an N-terminal domain bearing similarity to m7G methyltransferases involved in viral RNA capping, and a C-terminal domain containing a helicase-like motif (18, 19). BMV RNA2 encodes replication protein 2a, with domains characteristic of RNA-dependent RNA polymerases (20). BMV RNA3 contains two open reading frames (ORF) coding for protein 3a, necessary for cell-to-cell movement, and for a coat protein, essential for virion assembly and systemic movement throughout the host (21, 22). This coat protein is translated from subgenomic mRNA4, a viral mRNA that is transcribed during viral infections (14, 23).

Extensive work has demonstrated that BMV replication, subgenomic RNA transcription and translation can occur in yeast (Saccharomyces cerevisiae) when cells are transformed with the genes encoding the viral proteins (24, 25). Therefore, yeast is amenable to observing post-transcriptional processes that affect message stability, as mRNA decay pathways are well characterized. These include the normal turnover of mRNAs (26, 27) plus surveillance pathways that serve to recognize and degrade mRNAs with structural defects (28–30).

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³ The abbreviations used are: PAP, pokeweed antiviral protein; BMV, Brome mosaic virus; ORF, open reading frame; DTT, dithiothreitol; —EXT, minus yeast extract; PSY, MATa ade2-1 trp1-1 ura3-1 leu2-3 112 his3-11 15 can1-100; G6PD, glucose-6-phosphate dehydrogenase; PAPwt, wild-type PAP; PAPx, PAP with active-site mutant E176V.
PAP has been shown to reduce BMV replication and sub-genomic mRNA transcription in barley protoplasts and translation in a cell-free system (11, 13). To investigate the post-transcriptional effects of PAP on viral RNA and to distinguish these effects from inhibition of virus replication, we employed a non-replicating, inducible transcription system in yeast to track the activity of PAP on RNA3 alone. We show that PAP depurinated the RNA, which resulted in decreased viral protein synthesis and increased viral RNA degradation. Therefore, the antiviral activity of PAP includes these two consequences of RNA depurination in vivo.

EXPERIMENTAL PROCEDURES

Plasmids and Transformation into Yeast Strains—cDNAs of PAPwt (wild-type) or PAPx (active-site mutant E176V (31)) under the control of the GAL1 promoter, were transformed into Saccharomyces cerevisiae strains W303, also referred to as PSY (MATa ade2-1 trp1-1 ura3-1 leu2-3 112 his3-11 15 can1-100) and RP1674 (MATa his3Δ1 leu2Δ met15Δ ura3Δ), both used as wild-type strains. Empty vector, lacking a PAP cDNA, was also transformed as a negative control for PAP expression. BMV cDNA3 was amplified from the plasmid PB3TP8 (gift of Dr. C. C. Kao) using forward (5'-GGGGATC-CGTAAAATACCAACTAATTCTCG-3') and reverse (5'-GGGGGCGGCCGCTGGTCTCTTTAGAGATTTACAGTG-3') primers. BMV cDNA3 was cloned into the BamHI and NotI sites of pCM190 and transformed with PAPwt, PAPx, or vector control plasmids, resulting plasmid, KH042, was transformed into the BamHI and NotI sites of the uracil-based yeast-expression vector, pCM190, under control of a tetracycline-regulated promoter (32). The resulting plasmid, KH042, was transformed into yeast already transformed with PAPwt, PAPx, or vector control plasmids, using the polyethylene glycol/lithium acetate method (33). To identify the decay pathway of depurinated BMV RNA3, the viral cDNA, along with cDNAs of PAPwt or PAPx, were transformed into the deletion strains yRP2054 (xrn1:: KanMX4), yRP2071 (ski2:: KanMX4) and yRP2077 (upf1:: KanMX4). To investigate the involvement of the No-go decay pathway, the same plasmids were transformed into the deletion strains yRP2058 (dom34:: KanMX4 ski7:: KanMX4), yRP2059 (Met+ lys2Δ hbs1:: KanMX4 xrn1:: KanMX4), yRP2060 (Met+ lys2Δ dom34:: KanMX4 xrn1:: KanMX4), and yRP2074 (lys2Δ hbs1:: KanMX4 ski2:: KanMX4). All deletion strains were in the genetic background of yRP1674 and were provided by Dr. R. Parker. To observe if degradation directly affected RNA3 stability and translatability, a mutant cDNA3 was constructed by a series of overlapping PCRs to mutate the observed depurinated nucleotides in BMV RNA3 from purines to pyrimidines. The mutations, written as nucleotide change and (amino acid change), were as follows: A103C (I to I), A164T (T to S), A177C (K to Q), A371C (M to L), A374T (N to Y), A771T (D to V), A901C (E to A), A1197T (Y to F), A1634C (K to Q), A1672C (R to R), and A1953C (D to A). The resulting mutant cDNA3 was cloned into the BamHI and NotI sites of pCM190 and transformed, along with cDNAs of PAPwt or PAPx, into yeast strains PSY and RP1674.

PROTEIN EXPRESSION ANALYSIS—Transformed cells were grown to A600 of 0.4 in SD-leu-ura 2% raffinose with 50 μg/ml tetracycline to suppress BMV cDNA3 transcription. The cells were then pelleted and washed with dH2O before BMV RNA3 transcription was induced in SD-leu-ura 2% raffinose in the absence of tetracycline for 3 h. The cells were then pelleted, washed, and resuspended in SD-leu-ura 2% galactose, to induce transcription of either PAPwt or PAPx for 4 h. Cells were finally pelleted and lysed with glass beads in equal volume to pellet of protein complete buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol). An equal amount of total protein from each sample, as determined by the Bradford Assay, was separated through 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with polyclonal antibodies specific for either PAP (1:5000, provided by Dr. N. E. Turner), BMV protein 3a (1:1000, provided by Dr. K. Muse), or G6PD (glucose-6-phosphate dehydrogenase, 1:5000, Sigma). Proteins were visualized by chemiluminescence and exposure to x-ray film.

YEAST RNA ISOLATION AND NORTHERN BLOT ANALYSIS—Total RNA (15 μg) was isolated from 10-ml cultures induced to transcribe BMV RNA3 for 3 h prior to transcription activation of PAPwt or PAPx for 4 h. Total RNA was separated in a 7 M urea/4.5% acrylamide gel and transferred to nylon membrane. To detect BMV RNA3, the blot was probed with a nucleotide of ~200, radiolabeled negative-strand RNA complementary to the 3′-end of BMV RNA3 synthesized from PB3HE1 (130). The blot was also probed for G6PD mRNA, using a 5′-end-labeled cDNA primer (5′-TTAGGTACACACAGCGGC-3′) and RNAs were visualized by exposure to x-ray film. For time-course analysis of wild-type and mutant RNA3 in wild-type yeast or deletion strains, 100-ml cultures were induced to transcribe BMV RNA3 for 3 h. Cells were then switched to medium containing 50 μg/ml tetracycline (to suppress further RNA3 transcription) and 2% galactose (to induce PAPwt or PAPx transcription). This switch represented the zero time point of a 6-h time course during which 15-ml aliquots were removed for total RNA isolation. Equal amounts of total RNA (20 μg) were separated in a 7 M urea/4.5% acrylamide gel and transferred to nylon membrane. To detect BMV RNA3, the blots were probed with a radiolabeled RNA complementary to the 3′-end (as above) or 5′-end of BMV RNA3. The 5′-end of BMV RNA3 was amplified by PCR using forward (5′-GGGGAGCTGGCGGAACATTCTATTATCCACACATC-3′) and reverse (5′-GGGGAGATTCGTTGGCGGGGTTTCTAGTTATCGAC-CAG-3′) primers. The PCR product was cloned between the HindIII and EcoRI sites of KS pBluescript II vector. The cloned plasmid was linearized with HindIII to synthesize negative-strand radiolabeled transcripts complementary to the 5′-end of BMV RNA3. Blots were stripped and probed for 18 S rRNA using a 5′-end-labeled cDNA primer (5′-GGAAATTACCGCGGGCTCGTGGGCACCC-3′) to indicate RNA loading for each sample.

TRANSLATION REQUIREMENT FOR BMV RNA3 DEGRADATION—BMV RNA3 transcription was induced as described above in wild-type yeast and deletion strains for 3 h. The cells were then switched to medium containing 2% galactose to induce transcription of either PAPwt or PAPx for 1 h. Following this, BMV RNA3 transcription was inhibited with tetracycline (50 μg/ml), and cellular translation was repressed with cycloheximide (100 μg/ml). This switch represented the zero time point of a 6 h time course during which 15-ml aliquots were removed for total RNA isolation. An equal amount of total RNA (20 μg) was
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separated in a 7 M urea/4.5% acrylamide gel and transferred to nylon membrane. The blots were probed for BMV RNA3 and 18 S rRNA as described above.

RNA Half-life Measurements—Quantification of Northern blots from the time-course analysis was performed using Pharsys FX Plus Molecular Imager and Quantity One-4.6.1 one-dimensional analysis software (Bio-Rad). BMV RNA3 transcript levels at each time point (0, 1, 2, 4, and 6 h) were normalized with respect to the 18 S rRNA. The half-lives were determined by plotting the percentage of BMV RNA3 remaining at each time point versus the time after transcription repression.

RNA Binding and Immunoprecipitation—Yeast cells were grown in 10-ml cultures and induced to transcribe BMV RNA3 and express PAPwt or PAPx as described above. The cells were pelleted and resuspended in 4 ml of spheroplast buffer A (1 M sorbitol, 50 mM Tris-HCl, pH 7.5, 30 mM DTT, 15 mM β-mercaptoethanol, 1 mM MgCl₂, and spheroplasted with lyticase (200 units) for 30 min at 30 °C. The spheroplasts were then pelleted at 1000 g for 5 min and UV-cross-linked to 254 nm for 30 min at 4 °C. Cellular lysate was isolated from spheroplasts in protein complete buffer A as described above. PAP-containing complexes were isolated using polyclonal PAP antibody bound to protein-A-Sepharose beads for 2 h. Following incubation, the beads were washed five times with wash buffer (100 mM NaCl, 25 mM Hepes-KOH, pH 7.6, 0.1% Triton X-100). To elute the PAP-containing complexes from the beads, 100 µl of dH₂O was added, and the mixture was boiled for 10 min followed by incubation with proteinase K (100 ng) and 0.1% SDS for 1 h at 37 °C. The samples were extracted with phenol/chloroform, and nucleic acids were precipitated in ethanol. Reverse transcription, using random hexamers, followed by PCR was performed to detect (a) BMV RNA3 (Forward: 5′-CATTTTACACAAATCG-3′ and Reverse: 5′-CTTTCTACGCCCTTTTCAGG-3′ primers); and (b) G6PD mRNA (Forward: 5′-GGAGCAATGACTTTCAACA-3′ and Reverse: 5′-TTAGGTACACAGGGC-3′ primers) bound to PAPwt or PAPx. To test for in vitro binding of PAP to BMV RNA3, PAPwt (100 ng) was incubated with BMV RNA3 (500 ng) transcript at 30 °C for 10 min. Samples were then UV-cross-linked, and reverse transcription-PCR was performed as described in this section for in vivo binding.

Primer Extension of BMV RNA3—For analysis of depurination, PSY yeast cells were induced to transcribe BMV RNA3 and express PAPwt or PAPx as described above. Total RNA was isolated from the cells, treated with DNase (15 units) for 30 min, followed by phenol/chloroform extraction and precipitation in ethanol. For primer extension analysis, 2 µg of total yeast RNA was combined with 5 × 10⁵ cpm of 5′-end-labeled BMV cDNA3 probe (ten probes 200 nucleotides apart to cover the entire length of BMV RNA3; 2113 nucleotides) and extended with reverse transcriptase (100 units) for 20 min at 48 °C. Products of the reactions were denatured in formamide buffer and separated through a 7 M urea/6% acrylamide gel. To identify the location of BMV RNA3 depurination by PAPwt, deoxynucleotide sequencing of BMV cDNA3 was performed with the same primers used for primer extension analysis. Gels were dried against filter paper and exposed to x-ray film to visualize primer extension patterns and sequencing ladders. The same depurination sites of RNA3 were observed for four independent yeast transformations.

Polysome Profiles—Wild-type PSY and deletion mutant (dom34Δxrn1Δ and hbs1Δxrn1Δ) yeast cells were induced to transcribe BMV RNA3 and PAPwt or PAPx as described above. Following induction, cells were treated with cycloheximide (100 µg/ml) for 10 min, pelleted, and resuspended in lysis buffer (100 mM NaCl, 30 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 10 mM DTT, 10 mM phenylmethylsulfonyl fluoride, 50 µg/ml cycloheximide). Cells were pelleted again, resuspended in 1 ml of lysis buffer, and lysed by vortexing with glass beads. The samples were then centrifuged at 8,000 × g for 10 min, and supernatant was collected and centrifuged twice at 12,000 × g for 10 min. Lysate (14 A₂₆₀) was loaded on a step gradient of 10 and 35% sucrose prepared in 50 mM Tris-HCl, pH 7.0, 50 mM NH₄Cl, 12 mM MgCl₂, 1 mM DTT, 20 units of RNase inhibitor. To separate polysomes from messenger ribonuclear protein complexes, the gradient was centrifuged at 147,000 × g for 2 h at 4 °C. The polysome pellet was recovered from the bottom of the tube and applied to a 4 – 47% sucrose gradient. The gradient was centrifuged at 180,000 × g for 3 h at 4 °C. Fractions (28 of 150 µl) were collected at 4 °C and A₂₆₀ readings were recorded.

To determine if BMV RNA3 was associated with polysomes of PSY, each of two fractions was pooled sequentially, and isolated RNA was analyzed by Northern blot. BMV RNA3 was detected with a probe complementary to the 3′-end of the transcript, as described above for Northern blot analysis. To determine if RNA3 in polysome fractions was depurinated, isolated RNA from monosome and polysome fractions was pooled separately and analyzed by primer extension. Depurination was detected using the 5′-end-labeled BMV cDNA3 probe (5′-TGCTTAACACTCTACGAAATG-3′) complementary for the 3′-end of ORF3.

In Vitro Translation and Toe-printing Analysis—Translationally competent PSY extract was prepared using the liquid N₂-grinding protocol, column chromatography with Sephadex G-25, and nuclease treatment as described previously (34). The translation reaction mixture was prepared using 1.5-µl translation reaction components (10 mM ATP, 2.5 mM GTP, 250 mM creatine phosphate), 0.8 unit of creatine phosphokinase, 0.45 µl of 40× common buffer (400 mM Hepes-KOH, pH 7.6, 40 mM DTT), 1.8 µl of 10× variable buffer (34 mM magnesium acetate, 2.3 M potassium acetate), 0.18 µl of 1 mM amino acids, 3.6 units of RNase inhibitor, and 4 µl of water. For in vitro translation of RNA3, PAPwt or PAPx-treated wild-type or mutant BMV RNA3 (120 ng) (or dH₂O for minus RNA3 control) was added to the translation reaction mixture. The reaction was started by the addition of 10 µl of nuclease-treated cell extract and incubated at 25 °C for 2 h. Following incubation, the protein product was detected by immunoblot analysis with BMV protein 3a antibody (1:1000). For toe-print analysis, PAPwt- or PAPx-treated wild-type or mutant BMV RNA3 (120 ng) (or dH₂O for minus RNA3 control) was added to the translation reaction mixture. The reaction was started by the addition of 10 µl of nuclease-treated cell extract, and samples were incubated at 25 °C for 10 min. As controls for the elongation of ribosomes, cycloheximide (100 µg/ml) was added to some samples prior to
addition of yeast extract (t₀) or yeast extract was omitted (–EXT). The toe-printing reaction was initiated by the addition of 3 μl of translation reaction to 5.5 μl of annealing solution (1.25 μl of dH₂O, 2.0 μl of 5× reaction buffer, 1.0 μl of 0.1 M DTT, 1.0 μl of 2.5 mM dNTPs, 10 units of RNase inhibitor) and incubated at 55 °C for 2 min. The 5'-end-labeled BMV cDNA3 probe (5 × 10⁵ cpm) (5'-CTTCCACAGGCTTTTCAAGG-3') complementary to the 5'-end of ORF3, was then added, and the samples were incubated at 37 °C for 5 min. The reaction was supplemented with reverse transcriptase (50 units) and incubated at 37 °C for 30 min. Products of the reaction were denatured in formamide buffer and separated in a 7 M urea/6% polyacrylamide gel.

RESULTS

Expression of PAPwt and PAPx in Yeast—PSY cells were induced to express PAPwt or PAPx (active site mutant of PAP; 31) for 4 h, and expression was confirmed by immunoblot analysis (Fig. 1). The slower migrating band represents the 33-kDa immature form of the protein, which has been observed previously in yeast and shown to maintain activity (31), whereas the faster migrating band represents the 29-kDa mature, processed form. The results indicate similar levels of expression of PAPwt and PAPx in these cells.

BMV RNA3 Accumulation in Yeast Cells—Transcription of BMV RNA3 was induced for 3 h, followed by expression of PAPwt or PAPx for 4 h. Northern blot analysis indicated that the level of BMV RNA3 accumulation was ~3-fold lower in cells expressing PAPwt compared with cells expressing PAPx or cells transformed with empty vector (Fig. 2, A and C). The level of G6PD mRNA was not reduced in the presence of PAPwt and remained essentially constant in all samples (Fig. 2, B and C), indicating some specificity in the reduction of RNA3 levels by PAPwt. Moreover, the enzymatic activity of PAPwt was required for the decrease in viral RNA, as the active-site mutant PAPx did not cause a similar decrease in the level of BMV RNA3.

In Vivo Depurination of BMV RNA3—Results of UV-cross-linking and immunoprecipitation experiments show that BMV RNA3 was bound to PAPwt and PAPx in vivo. Even though PAPx could not depurinate, it retained the ability to bind viral RNA. The viral RNA was not observed in precipitates of vector control cells, indicating PAP-dependent immunoprecipitation of BMV RNA3 (Fig. 3A). In addition, in vitro UV-cross-linking of PAPwt with BMV RNA3, followed by immunoprecipitation, confirmed that PAPwt could bind BMV RNA3 directly in the absence of cellular proteins (Fig. 3A). To verify some specificity of PAP binding to BMV RNA3, similar experiments were performed to determine if PAPwt or PAPx was bound to G6PD mRNA. The results demonstrate that neither protein targeted this message in vivo (Fig. 3B).

Depurination of BMV RNA3 by PAP—To identify the nucleotides of BMV RNA3 targeted by PAP in vivo, depurination sites on the viral RNA were examined in PAPwt-expressing PSY cells. The primer extension patterns indicated depurination of RNA3 within ORFs 3 and 4, plus depurination within the intergenic region between these ORFs and in the 3' untranslated region. No depurination was noted in the 5' untranslated region. Fig. 4A maps these sites relative to the coding and regulatory regions of BMV RNA3 and representative examples of primer extension of sites in all four targeted regions are shown (Fig. 4B). Bands present in all lanes represented nonspecific, strong stops for the reverse transcriptase, and only bands
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**FIGURE 3. Binding of PAPwt and PAPx to BMV RNA3 and G6PD mRNA.**

A, for the *in vitro* sample, PAPwt (100 ng) was incubated with BMV RNA3 transcript (500 ng), followed by UV-cross-linking and immunoprecipitation of PAPwt. For the *in vivo* samples, transcription of BMV RNA3 was induced in PSY cells for 3 h, followed by PAP induction for 4 h. Cells were transformed with empty vector (VC) as a control for PAP expression. The cells were then spheroplasted and UV-cross-linked, and PAP was immunoprecipitated from the lysate of spheroplasts. Nucleic acids were extracted from the precipitate and used as template for reverse transcription and PCR with specific BMV DNA3 primers. A plasmid encoding BMV DNA3 as PCR template was used as a positive control (+ctrl), and a PCR reaction without template DNA was used as a negative control (−ctrl). B, samples were prepared as described in A, and nucleic acids extracted from the precipitate were used as template for reverse transcription and PCR with specific G6PD primers. A plasmid encoding G6PD as PCR template was used as a positive control (+ctrl), and a PCR reaction without template DNA was used as a negative control (−ctrl).

**FIGURE 4. Depurination of BMV RNA3.** PSY yeast cells were induced to transcribe BMV RNA3 for 3 h, followed by induction of PAPwt or PAPx for 4 h. Following induction, total RNA was isolated and extended with reverse transcriptase using BMV RNA3 primers distributed over the length of the viral RNA. The resulting cDNAs from each extension were separated through a 7 M urea/6% acrylamide gel. The same primers were used to determine the position of depurination sites by deoxynucleotide sequencing of BMV DNA3. A, locations of ORFs and regulatory regions in BMV RNA3 with observed depurination sites indicated by their nucleotide number. B, representative depurination sites from within ORF3, the intergenic region (IGR), ORF4, and the 3′-untranslated region.

whether these RNAs were fit templates for translation. To determine whether depurinated RNA3 caused ribosomes to stall, RNA3 transcripts were treated with PAPwt or PAPx and used as template for *in vitro* translation followed by toe-printing analysis. A primer complementary to the 5′ portion of RNA3 and downstream of a known depurination site near the start codon of ORF3 was chosen for primer extension, as it was anticipated that ribosomes may stall at the first depurination site encountered during elongation. Negative controls for the elongation of ribosomes are indicated by $t_0$ and $-\text{EXT}$. Addition of cycloheximide to the RNA template prior to adding yeast extract ($t_0$) should allow ribosomes to load at the initiation codon but not to elongate. Similarly, reactions lacking yeast extract ($-\text{EXT}$) will illustrate the extension pattern of the reverse transcriptase in the absence of ribosomes. Within the control samples treated with PAPwt, the reverse transcriptase stalled at the expected depurination site (A103) and also stalled at several sites downstream of this nucleotide (Fig. 6A). We interpret these less intense downstream bands as natural stalls for the reverse transcriptase, perhaps caused by conformational changes to the RNA due to depurination at A103, because these stalls were not visible in RNA treated with the non-depurinating mutant PAPx. Samples containing yeast extract were permitted to translate for 10 min ($t_{10}$) prior to reverse transcription. The extension pattern from PAPwt-treated RNA3 unique to PAPwt were scored as depurinations. Intensity differences among depurination bands indicated that not all RNA3 molecules were depurinated at all sites. As expected in the PAPx and vector control cells, no corresponding depurination bands were observed.

**Polysome Association of RNA3**—To determine whether BMV RNA3 was associated with polysomes in PAPwt-expressing PSY cells, polysomes were separated from non-translating ribosomal protein complexes and analyzed by Northern blot for the presence of RNA3. The first notable difference in profiles was the lower polysome peaks in PAPwt-expressing cells compared with the PAPx and VC cells (Fig. 5A). This decrease could be due to fewer ribosomes engaged in translation, either because PAPwt depurinated cellular messages apart from the viral BMV RNA3, or that depurinated rRNA enhanced the degradation of ribosomes. The fractions contained RNA3 (Fig. 5B); however, the RNA3 associated with polysomes and monosomes from PAPwt-expressing cells was depurinated. For example, a probe specific to the 3′ portion of ORF3 confirmed the same depurination site, A901, initially mapped from total RNA of PAPwt-expressing cells (Fig. 5C).

**Ribosome Stalling at Site of Depurination**—The presence of depurinated RNA3 in polysome fractions raised the question of...
illustrates a termination site indicative of ribosome stalling. Deoxynucleotide sequencing of the viral DNA3 with the same primer showed that ribosomes stalled ~10 nucleotides downstream of the known depurination site at A103, placing the depurinated nucleotide at the wobble base of the codon within the A-site of the ribosome (Fig. 6B). The termination site was not observed with template treated with PAPx, the inactive mutant; therefore, we conclude that depurination of RNA3 caused ribosomal elongation arrest. Additional downstream termination bands were observed in samples treated with PAPwt that may represent the presence of factors associated with the ribosomes. Alternatively, these additional bands may be caused by ribosomes pausing at RNA structures introduced by treatment with PAPwt, because they were absent from samples treated with PAPx.

To confirm that depurination of BMV RNA3 was directly responsible for stalling of ribosomes in vitro, the toe-print analysis was repeated using a mutant form of RNA3, one in which every observed depurinated adenine was changed to a pyrimidine. Such an RNA template should not be targeted by PAP. No depurination band was observed at nucleotide position A103, mutated to Cys-103, nor was a ribosome stall signal detected downstream of this site, indicating that depurination of RNA3 was required for ribosome stalling (Fig. 6A). Differences in extension pattern between wild-type and mutant RNA3 may be due to changes in RNA secondary structure introduced by the substitutions of purines for pyrimidines along the length of RNA3.

Ribosome stalling suggested that less than full-length protein 3a would be synthesized from depurinated RNA3. To test for
the incomplete synthesis of protein 3a (Fig. 6). Translation of mutant RNA3 treated with PAPwt suggests RNA3. Moreover, the lack of shorter proteins observed from containing PAPx-treated template would argue that they are strains bearing deletions of proteins required for 5' cleavage, or as a result of internal cleavage (fragments indicated by arrowheads). Similarly, fragments were seen in the ski2Δ strain when RNA3 was visualized with a probe specific to the 3'-end of RNA3, suggesting that the fragments either arose by decapping, or as a result of endonucleolytic cleavage (fragments indicated by arrowheads). To distinguish the two possibilities, either cleavage or exonucleolytic decay, we tested whether depurinated RNA3 was template for the No-go decay pathway, in which ribosome stalling on an RNA (triggered by depurination in this case) would result in endonucleolytic cleavage and subsequent degradation from the newly exposed 5'- and 3'-ends (29). To this end, we transcribed RNA3 and expressed PAPwt or PAPx in strains deficient for the nuclease, Dom34p, and associated protein, Hbs1p, involved in No-go decay. North-

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| Yeast strain | Half-lives of BMV RNA3 | PAPwt | PAPx | VC |
|--------------|------------------------|-------|------|----|
| Wild-type RNA3 |                        |       |      |    |
| PSY (wt) − CHX | 2.1 ± 0.06             | 3.0 ± 0.04 | 3.1 ± 0.03 |
| RP1674 (wt) − CHX | 2.2 ± 0.08             | 3.0 ± 0.06 | 3.0 ± 0.03 |
| PSY (wt) + CHX | 3.1 ± 0.06             | 3.1 ± 0.09 | 3.1 ± 0.05 |
| RP1674 (wt) + CHX | 3.2 ± 0.07             | 3.1 ± 0.1 | 3.0 ± 0.07 |
| xrn1Δ | 3.0 ± 0.06             | 3.4 ± 0.09 | 3.5 ± 0.05 |
| ski2Δ | 2.9 ± 0.04             | 3.1 ± 0.1 | 3.1 ± 0.07 |
| upf1Δ | 2.2 ± 0.08             | 3.2 ± 0.07 | 3.0 ± 0.04 |
| dom34Δ, ski2Δ | 4.3 ± 0.10             | 3.2 ± 0.09 | 3.4 ± 0.12 |
| dom34Δ, xrn1Δ | 4.9 ± 0.08             | 3.5 ± 0.13 | 3.2 ± 0.11 |
| hbs1Δ, ski2Δ | 4.0 ± 0.21             | 3.2 ± 0.13 | 3.2 ± 0.11 |
| hbs1Δ, xrn1Δ | 3.9 ± 0.12             | 3.2 ± 0.11 | 3.3 ± 0.14 |
| Mutant RNA3 |                        |       |      |    |
| PSY (wt) | 2.8 ± 0.02             | 3.0 ± 0.01 | 3.0 ± 0.06 |
| RP1674 (wt) | 2.7 ± 0.07             | 3.1 ± 0.04 | 3.0 ± 0.05 |
ern blots of RNA3 decay profiles in double deletion strains expressing PAPwt indicated a lack of the fragments seen previously in the xrnlΔ and ski2Δ strains (Fig. 7), suggesting that the endonucleolytic activity of Dom34p and associated Hbs1p contributed to the fragmentation of depurinated BMV RNA3. In the double deletion strain dom34Δski7Δ, Ski7p was deleted rather than Ski2p; however, both proteins are involved in the exosomal decay pathway. Namely, Ski7p is an adapter protein that binds to the ribosomal A site to release the transcript, and then recruits the exosome and the associated SKI complex, of which Ski2p is a component, to degrade the transcript in the 3'→5' direction (35, 36). Therefore, either the absence of Ski7p or Ski2p will impair 3'→5' decay. Larger fragments observed with 5' probing of RNA3 in the xrnlΔ strain expressing PAPx or vector control were also not seen in the dom34Δ and hbs1Δ strains, for reasons that are not well understood. An RNA fragment detected with the 3'→5' probe in the upf1Δ strain also suggests a role for this protein in the normal degradation of RNA3, given its presence in PAPx-expressing and vector control cells, even though the absence of Upf1p did not change the half-life of RNA3. Some heterogeneity existed in the size of fragments observed in the upf1Δ strain, which may be due to trimming by exonucleases.

The RNA3 half-life in the dom34ΔxrnlΔ or hbs1ΔxrnlΔ strain expressing PAPx or vector control was similar to the RNA3 half-life in the single deletion strain xrnlΔ, suggesting that normal decay of RNA3 does not involve Dom34p or Hbs1p. Our interpretation that the No-go decay pathway is involved in the degradation of depurinated RNA3 is supported by the observation that the stability of RNA3 in PAPwt-expressing cells of the double deletion strains deficient in Dom34p or Hbs1p was substantially greater than in the single deletion strains xrnlΔ or ski2Δ (Table 1). In cells expressing PAPwt, the RNA3 half-life in the double deletion strains exceeded that in cells expressing PAPx or vector control. This extended half-life may be due to the fact that PAPwt-expressing cells are not translationally competent because ribosomes are stalling at depurination sites. We hypothesize that an intermediate in the decay pathway is trapped by ribosomes that are not efficiently cleared from RNA3. Support for this idea comes from increased RNA3 stability in PAPwt-expressing cells treated with cycloheximide (+CHX, Table 1), indicating that active translation is required for efficient turnover of depurinated RNA3.

Our observation that the amount of polysomes was diminished in PAPwt-expressing cells suggested that other cellular messages, apart from BMV RNA3, could be depurinated and also targeted for degradation, thereby decreasing the number of mRNAs being translated. To test this possibility, polysomes were isolated from the double deletion strains dom34ΔxrnlΔ and hbs1ΔxrnlΔ. Profiles illustrate an increase in polysome peaks from PAPwt-expressing cells (Fig. 8), indicating that other cellular messages may also be targeted by PAP and degraded by the No-go pathway. However, polysome peaks from PAPwt-expressing cells did not reach the levels observed in PAPx and VC cells, suggesting fewer ribosomes in these cells, a possibility that is also supported by consistently lower 40 S and 60 S peaks. Therefore, depurination of both cellular mRNAs and rRNA may have contributed to the decreased polysome peaks observed in PAPwt-expressing cells.

Translation Levels of BMV Protein 3a in Vivo—The reduced half-life of wild-type BMV RNA3 in PAPwt-expressing cells and evidence for the stalling of ribosomes at depurination sites suggested that the level of protein 3a would be decreased. Cell lysates from PAPwt- or PAPx-expressing cells were analyzed by immunoblot for the presence of BMV protein 3a and showed an ~4-fold difference in the level of movement protein in cells expressing PAPwt relative to vector control cells (Fig. 9, A and C). The level of protein 3a did not decrease in PAPx-expressing cells, indicating that inhibition of protein accumulation was due to the enzyme activity of PAPwt. To test the direct effect of RNA3 depurination on the level of protein 3a, mutant RNA3 was expressed with PAPwt or PAPx in PSY cells. Protein product from mutant RNA3 was 3-fold greater than from wild-type RNA3 (Fig. 9, A and C). As an indicator of specificity for this decrease, samples were probed for G6PD, shown previously to be unaffected by PAP (Fig. 9B (37)). Because the levels of G6PD remained constant, our observations suggest that PAPwt was able to target specific templates for depurination, resulting in reduced efficiency of translation.

DISCUSSION

The antiviral activity of some ribosome-inactivating proteins was initially attributed to their toxicity; the premise being that rRNA depurination would cause host cell death and thereby

FIGURE 8. Representative polysome profiles of yeast deletion strains dom34ΔxrnlΔ (A) and hbs1ΔxrnlΔ (B) induced to transcribe RNA3 for 3 h prior to PAPwt or PAPx induction for 4 h. Cell lysates were centrifuged through a 10 and 35% sucrose cushion to separate mRNPs from ribosomes. Ribosome pellets were separated by 7–47% gradient sucrose centrifugation and the absorbance of collected fractions was measured at A260.
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A

Wild-type 3a

Mutant 3a

Protein 3a antibody (1:1000).

B

G6PD antibody (1:5000).

C

Protein levels relative to vector control.

FIGURE 9. Effect of PAP on level of BMV 3a protein. A, PSY yeast cells were induced to transcribe wild-type or mutant BMV RNA3 for 3 h followed by PAPwt or PAPx induction for 4 h. Total cellular proteins (12 μg) were separated in 12% SDS-PAGE, transferred to nitrocellulose, and probed with BMV protein 3a antibody (1:1000). B, the same samples probed with G6PD antibody (1:5000). C, quantitative representation by densitometric analysis of wild-type or mutant BMV protein 3a (gray bars) and G6PD (white bars) levels in PAPwt- or PAPx-expressing cells relative to vector control cells. Bars represent means ± S.E. for three independent experiments.

limit virus proliferation (38, 39). This scenario has since been challenged by the description of PAP mutants that maintain antiviral activity in the absence of rRNA depurination (9, 10). In a previous study, we reported that incubation of PAP with BMV RNAs in vitro, followed by aniline treatment, resulted in cleavage of the RNAs, indicating that PAP depurinated substrates other than rRNA (11). However, the depurination sites were not determined. In the current work, we extend these initial results by showing that PAP binds to viral RNA and depurinates specific nucleotides, which is the first demonstration that a viral message can be directly targeted in vivo by any antiviral ribosome-inactivating protein. How PAP selects viral RNA for depurination and the potential role of sequence or RNA conformation in determining a depurination site are currently under study. With depurination observed in vivo, this work focused on the post-transcriptional effects of this RNA modification.

The antiviral activity of PAP could be manifest at different stages during the virus life-cycle. Specifically, PAP may affect the synthesis or stability of viral RNAs and/or translation of viral proteins. We have demonstrated previously that treatment of BMV RNAs with PAP, prior to transfection into barley protoplasts, reduced the accumulation of these RNAs (13). The decreased amount of viral RNA was due to inhibition of replication, that is, the synthesis of new copies of genomic RNAs 1, 2, and 3 via a negative-strand intermediate, and inhibition of transcription, which is the synthesis of subgenomic RNA4 during infection (Fig. 10A). The current non-replicating system in yeast allowed us to observe whether depurination specifically affected the stability of the viral RNA and its fitness for translation. Our results show that depurination of BMV RNA3 caused ribosomes to stall during elongation, which resulted in decreased protein 3a synthesis and accelerated RNA3 degradation (Fig. 10B).

Our observation that PAPwt-expressing cells contained less RNA3 relative to PAPx-expressing cells suggested that depurination decreased the stability of the viral RNA, a view that was supported by the shorter half-life of RNA3. Base excision repair of DNA in eukaryotes involves endonucleolytic cleavage of the sugar–phosphate backbone upstream of the missing base, insertion of an intact nucleotide and removal of the sugar–phosphate fragment by polymerase β, followed by ligation of the DNA (40, 41). Although the process for replacement of abasic nucleotides in DNA is known, no such repair mechanism for RNA has been described; therefore, we hypothesized that RNA3 depurinated by PAP was likely subject to accelerated degradation. The mRNA decay pathways in yeast are well characterized, and several comprehensive reviews detail normal decay pathways, with their deadenylation-dependent and independent mechanisms (26, 42), and the mRNA surveillance pathways, which include nonsense-mediated decay and non-stop decay (43, 44). The recently described No-go decay pathway belongs to the latter category of mRNA surveillance and occurs when ribosomes stall during elongation, confronted by a stable stem-loop, a pseudoknot or a rare codon. Ribosome stalling triggers endonucleolytic cleavage near the stall site and subsequent exonucleolytic decay from the cut 5′ and 3′-ends (29). Among the known mRNA degradation pathways in yeast, No-go decay is the only one to involve endonucleolytic cleavage of the mRNA. The significant increase in RNA3 half-life in PAPwt-expressing cells deficient in the endonuclease Dom34p and associated Hbs1p involved in No-go decay suggests a role for these proteins in degradation of depurinated RNA. Comparison of RNA3
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fragmentation patterns in PAPwt-expressing strains deficient for Xrn1p or Ski2p, relative to double deletion strains also lacking the Dom34p or associated Hbs1p, suggested that depurinated viral RNA was a template for endonucleolytic cleavage. Moreover, the presence of depurinated RNA3 in polysome fractions, along with toe-print analysis indicating ribosome stalling, support involvement of No-go decay; that is, aberrant RNA is detected by a halt in translation elongation. The increased stability of RNA3 in PAPwt-expressing cells treated with cycloheximide is consistent with the requirement for actively translating ribosomes to recognize depurinated RNA and trigger its degradation.

The mechanism by which Dom34p and Hbs1p catalyze the decay of depurinated RNA is not known. We show that ribosomes stalled with a depurinated nucleotide at the A-site, and we hypothesize that this depurination would prevent the proper accommodation of a cognate tRNA. The recent crystal structure of Dom34p shows shape similarity to translation release factor eRF3 (49, 50). Unlike eRF1 however, Dom34p does not recognize stop codons and does not contain the conserved sequence necessary for peptidyl-tRNA hydrolysis, but it does contain an RNA-binding domain with endonuclease activity (47, 48). The associated Hbs1p is a G protein that binds Dom34p (48) and has sequence similarity to translation release factor eRF3 (49, 50). We posit that a ribosome stalled with a depurinated nucleotide within a codon at the A-site would allow the binding of Dom34p/Hbs1p at or near this site; Dom34p would cleave the mRNA with the assistance of Hbs1p, producing cut 5’- and 3’-ends as templates for subsequent exonucleolytic degradation (Fig. 10B).

Depurination within ORF3 contributed to the decrease of protein 3a product, due to both accelerated RNA3 decay and translation elongation arrest. We postulate that the observed ribosome stalling, with the depurinated nucleotide at the wobble base of a codon in the ribosomal A-site, would interfere with tRNA base-pairing and subsequent peptidyl transfer; hence, protein synthesis would stop. Without the binding of aminoacyl-tRNA to the A-site, the mRNA would be susceptible to cleavage by Dom34p and Hbs1p, which would likely cause the separation of ribosomal subunits and premature release of the nascent peptide (Fig. 10B). Support for this idea comes from the presence of truncated 3a protein synthesized in vitro from PAPwt-treated RNA3 in yeast extract and decreased amounts of intact 3a protein in PAPwt-expressing cells.

In the current study, we have expressed both PAP and BMV RNA3 in yeast to focus on viral RNA stability and translation in the presence of this antiviral protein. We demonstrate that depurination caused ribosomes to stall, which resulted in accelerated decay of the viral RNA and decreased translation of viral protein. Depurinated RNA was recognized by actively translating ribosomes and was degraded by components of the No-go decay pathway. Therefore, post-transcriptional effects of depurination, namely decreased stability and translational fitness of the viral RNA, contribute to the antiviral activity of PAP.
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