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Mutagenesis of Amino Acids at Two Tomato Ringspot Nepovirus Cleavage Sites: Effect on Proteolytic Processing *in cis* and *in trans* by the 3C-like Protease

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Tomato ringspot nepovirus (ToRSV) encodes two polyproteins that are processed by a 3C-like protease at specific cleavage sites. Analysis of ToRSV cleavage sites identified previously and in this study revealed that cleavage occurs at conserved Q/(G or S) dipeptides. In addition, a Cys or Val is found in the −2 position. Amino acid substitutions were introduced in the −6 to +1 positions of two ToRSV cleavage sites: the cleavage site between the protease and putative RNA-dependent RNA polymerase, which is processed *in cis*, and the cleavage site at the N-terminus of the movement protein, which is cleaved *in trans*. The effect of the mutations on proteolytic processing at these sites was tested using *in vitro* translation systems. Substitution of conserved amino acids at the −2, −1, and +1 positions resulted in a significant reduction in proteolytic processing at both cleavage sites. The effects of individual substitutions were stronger on the cleavage site processed *in trans* than on the one processed *in cis*. The cleavage site specificity of the ToRSV protease is discussed in comparison to that of related proteases.

INTRODUCTION

Nepoviruses have a bipartite genome, consisting of two molecules of positive-sense single-stranded RNA (RNA-1 and RNA-2) (Sanfaçon, 1995; Mayo and Robinson, 1996). Each RNA encodes a polyprotein, which is processed by a viral protease to release the mature proteins. Nepoviruses have been divided into three subgroups (subgroups a, b, and c) (Mayo and Robinson, 1996). Tomato ringspot nepovirus (ToRSV) is the only member of nepovirus subgroup c for which the entire nucleotide sequence has been determined (Rott *et al.,* 1991, 1995). The P1 polyprotein (encoded by RNA-1) contains the domains for a putative RNA-dependent RNA polymerase (Pol), a protease (Pro), the VPg protein, and a putative NTP-binding protein (Rott *et al.,* 1995; Wang *et al.,* 1999). The P2 polyprotein (encoded by RNA-2) includes the domains for the coat protein (CP) and the movement protein (MP) (Rott *et al.,* 1991; Wieczorek and Sanfaçon, 1993; Hans and Sanfaçon, 1995). We have previously shown that the ToRSV protease is responsible for cleavage between the MP and CP domains on P2 (Hans and Sanfaçon, 1995) and between the putative NTP-binding protein, VPg, Pro, and Pol domains on P1 (Wang *et al.,* 1999). The ToRSV protease is related to the 3C-like proteases of picornaviruses, nepoviruses, comoviruses, and potyviruses (Gorbalenya *et al.,* 1989). The three-dimensional structure of three picornavirus 3C-proteases is similar to that of chymotrypsin (Allaire *et al.,* 1994; Matthews *et al.,* 1994; Mosimann *et al.,* 1997), and the catalytic triad consists of His, Glu (or Asp), and Cys. The substrate binding pocket of 3C-like proteases from picornaviruses, potyviruses, comoviruses, and ToRSV contains a His residue (Bazan and Fletterick, 1990), which was shown to interact with a conserved Gln in the −1 position of the cleavage sites (Allaire *et al.,* 1994; Matthews *et al.,* 1994; Mosimann *et al.,* 1997). Proteases of nepovirus subgroup a/b do not include this His and recognize different cleavage sites (Margis and Pinck, 1992; Fig. 1).

The 3C-like proteases from picornaviruses, comoviruses, and potyviruses recognize cleavage sites with a consensus sequence of (−1)(E or Q)/(G, S, or M)(+1) (Bazan and Fletterick, 1990; Dewalt *et al.,* 1989; Gorbalenya *et al.,* 1989; see Fig. 1). Amino acid residues other than the amino acid pair at the cleaved peptide bond are also involved in the cleavage site specificity. Conserved amino acids are found in the −6, −4, and −3 positions of the tobacco etch potyvirus (TEV) cleavage sites, in the −4 position of several picornavirus cleavage sites, and in the −4 and −2 positions of cowpea mosaic comovirus (CPMV) cleavage sites (see Fig. 1). Site-directed mutagenesis studies have confirmed the importance of these conserved amino acids for proteolytic processing at the TEV, hepatitis A virus (HAV), human rhinovirus, and poliovirus cleavage sites (Dougherty *et al.,* 1988; Long *et al.,* 1989; Pallai *et al.,* 1989; Cordingley *et al.,* 1990; Blair

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NEPOVIRUS SUBGROUP C			
TomRSV	X-MP	TRSN*C*Q/S	This study
	Pro-Pol	SFAP*C*Q/S	Wang *et al.* (1999)
	MP-CP	RNSS*V*Q/G	Hans and Sanfacon(1995)
	VPg-Pro	RPQS*V*Q/G	Wang *et al.* (1999)
	NTB-VPg	GKMT*V*Q/S	Wang *et al.* (1999)
BLMV	MP-CP	RFTT*C*N/S	Bacher *et al.* (1994)
CLRV	MP-CP	INLP*L*Q/S	Scott *et al.* (1993)
BRAV	MP-CP	RFST*C*D/S	Latvala *et al.* (1998)
NEPOVIRUS SUBGROUP A/B			
ArMV	MP-CP	MRTTTR/G	Bertioli *et al.* (1991)
	-MP	STSV*C*C/A	Loudes *et al.* (1995)
TBRV	MP-CP	SLEN*L*K/A	Demangeat *et al.*(1992)
	NTB-VPg	SAVD*I*K/A	Hemmer *et al.* (1995)
	VPg-Pro	RYAYAK/S	Hemmer *et al.* (1995)
GCMV	MP-CP	SETN*L*R/A	Brault *et al.* (1989)
RRSV	MP-CP	ENVPGC/A	Block *et al.* (1992)
GFLV	MP-CP	LSST*V*R/G	Serghini *et al.* (1990)
	28K-MP	STSV*C*C/A	Margis *et al.* (1993)
	NTB-VPg	NASIPC/S	Pinck *et al.* (1991)
	VPg-Pro	ISKIRG/E	Pinck *et al.* (1991)
	Pro-Pol	SSSF*I*R/G	Margis *et al.* (1991)
TRSV	MP-CP	MC/A	Buckley *et al.* (1993)

COMOVIRUS CONSENSUS			
CPMV	XXAXAQ/S P G M		Wellink *et al.* (1986)
PICORNAVIRUS CONSENSUS			
Polio	XXAXXQ/G S		Nicklin *et al.* (1986)
Rhinovirus	XXVXXQ/G T E/S A		Long *et al.* (1989)
HAV	XXVXXQ/G L E/S I		Jewell *et al.* (1992)
POTYVIRUS CONSENSUS			
TEV	EXIYXQ/S L G V		Dougherty *et al.* (1988)
TuMV	XXVXHQ/S A T		Kim *et al.* (1996)

FIG. 1. Comparison of cleavage sites from nepoviruses (subgroups a, b, and c), comoviruses, picornaviruses, and potyviruses. Amino acid sequences from the −6 to the +1 positions of nepovirus cleavage sites are compared with the consensus sequences of cleavage sites from representative related viruses. Only cleavage sites that have been confirmed by direct amino acid sequencing are shown. Amino acids consistent with the picorna-like dipeptide cleavage site consensus in the +1 and −1 positions are underlined. Cys, Val, Leu, and Ile residues in the −2 position of nepovirus cleavage sites are in italics. BLMV indicates blueberry leaf mottle virus; CLRV, cherry leaf roll virus; BRAV, blackcurrant reversion associated virus; ArMV, arabis mosaic virus; TBRV, tomato black ring virus; GCMV, grapevine chrome mosaic virus; RRSV, raspberry ringspot nepovirus; GFLV, grapevine fanleaf virus; TRSV, tobacco ringspot virus; CPMV, cowpea mosaic virus; Polio, poliovirus; HAV, hepatitis A virus; TEV, tobacco etch virus; and TuMV, turnip mosaic virus.

and Semler, 1991; Jewell *et al.,* 1992). Four ToRSV cleavage sites have been identified so far (Hans and Sanfaçon, 1995; Wang *et al.,* 1999). Cleavage occurred at Q/G and Q/S dipeptides, and a Cys or Val residue was found at the −2 position (Wang *et al.,* 1999; see Fig. 1). Other amino acids did not appear to be conserved. Substitution of the conserved Gln at the −1 position of the ToRSV Pro-Pol cleavage site with Ala prevented processing at this site (Wang *et al.,* 1999). The importance of conserved amino acids on proteolytic processing of cleavage sites from nepoviruses and comoviruses has not been systematically studied.

In this report, a new cleavage site at the N-terminus of the ToRSV movement protein (X-MP cleavage site) was identified that contained the conserved amino acids identified for other ToRSV cleavage sites. A site-directed mutagenesis study was conducted on two cleavage sites to test the importance of amino acids from the −6 to the +1 position in determining a functional ToRSV cleavage site.

RESULTS

Characterization of the cleavage site between the protease and polymerase

As a first step toward the study of the cleavage site specificity of the protease, proteolytic processing at the Pro-Pol cleavage site was characterized. The Pro-Pol cleavage site has been previously identified to be Q^{1486}/S (Wang *et al.,* 1999). Cleavage at this site *in vitro* was demonstrated using a precursor (VPg-Pro-N-Pol-II) that included the entire VPg and Pro domains and the N-terminal portion of the polymerase (Wang *et al.,* 1999). Cleavage of the VPg-Pro-N-Pol-II precursor released two products: the VPg-Pro intermediate precursor and the N-Pol fragment, which was very unstable and often not detectable. The VPg-Pro intermediate did not undergo further cleavage under the conditions used. To stabilize the N-Pol fragment and to improve the quantification of processing at the Pro-Pol cleavage site, a new plasmid was constructed that contained the chloramphenicol acetyl transferase (CAT) coding region fused in frame at the C-terminal end of the polymerase fragment (plasmid pT7VPg-Pro-N-Pol-CAT; Fig. 2a). We have shown previously that the presence of the VPg protein on the precursor enhanced cleavage at the Pro-Pol site (Wang *et al.,* 1999). The VPg protein was therefore included on the VPg-Pro-N-Pol-CAT precursor to ensure optimal processing at the Pro-Pol site. As a control, a mutant derivative was also constructed that contained a mutation in the putative catalytic triad of the protease (plasmid pT7VPg-Pro-N-Pol^{H1283D}-CAT, His^{1283} mutated to Asp). This mutation was previously shown to inactivate the protease (Hans and Sanfaçon, 1995; Wang *et al.,* 1999). Translation

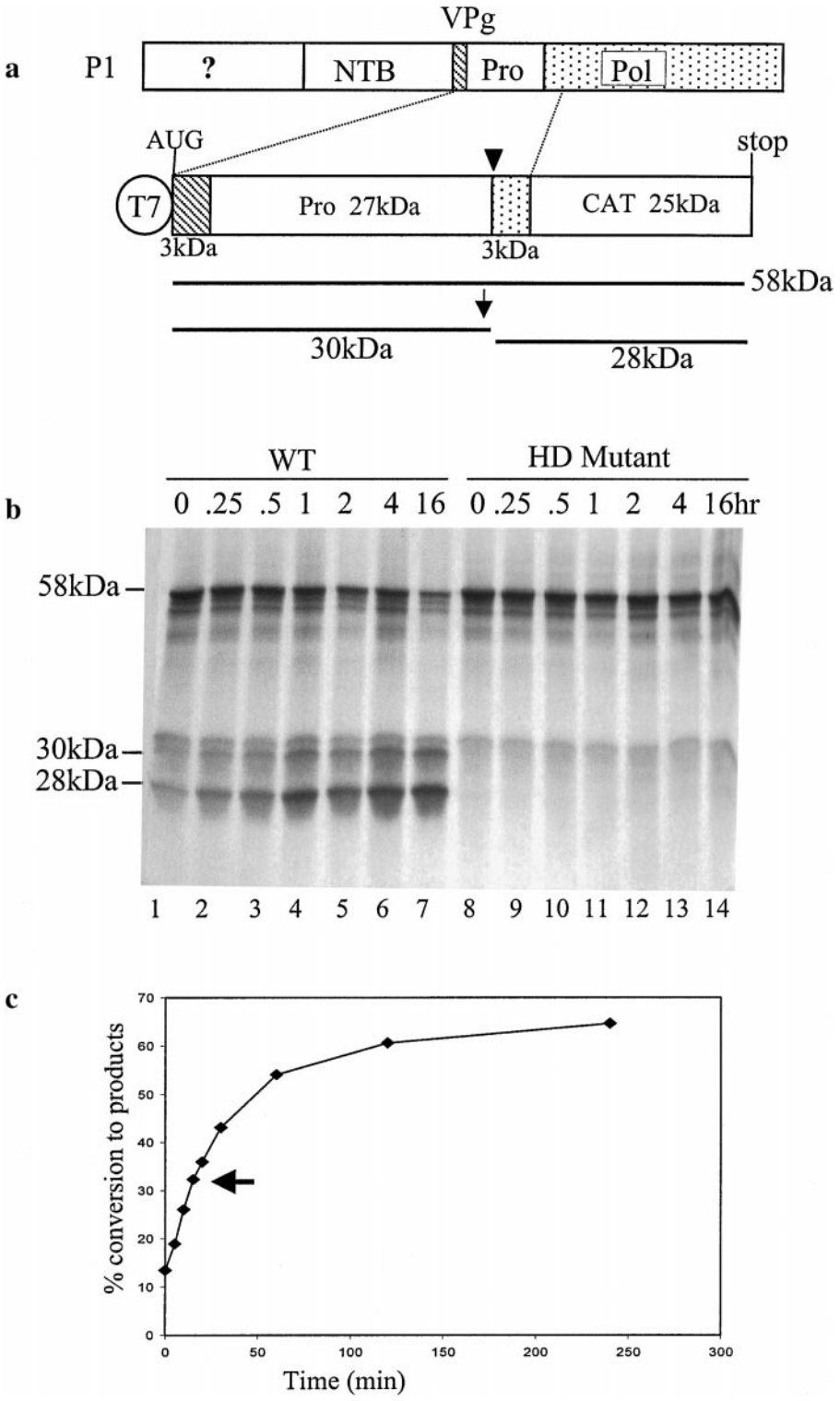


FIG. 2. Construction of the VPg-Pro-N-Pol-CAT precursor and proteolytic processing at the Pro-Pol cleavage site *in vitro*. (a) Schematic representation of the cDNA clone encoding the VPg-Pro-N-Pol-CAT precursor. The entire P1 polyprotein is represented on the top of the figure, and plasmid pT7VPg-Pro-N-Pol-CAT is shown below. The coding regions for the different ToRSV protein domains and for the CAT protein are shown along with the predicted molecular mass of each domain. The Pro-Pol cleavage site is indicated by the arrowhead. The predicted translation products are shown before (58-kDa precursor) and after (30- and 28-kDa cleaved products) processing by the endogenous protease. (b) Processing of VPg-Pro-N-Pol-CAT precursor *in vitro*. Processing was tested in precursors containing a wild-type protease (WT) or a protease mutated in the catalytic triad (HD mutant) as described in Materials and Methods. The translation products were separated on a 12% SDS–polyacrylamide gel. The time of incubation of the different samples is indicated above each lane (hr indicates hours). The expected position of the precursor and cleaved products are indicated on the left side of the gel. (c) Time course of processing of the VPg-Pro-N-Pol-CAT precursor to the 28-kDa product. The arrow indicates the percent of conversion at the 15-min time point.

products were generated using a coupled transcription–translation rabbit reticulocyte system in the presence of [^{35}S]methionine (see Materials and Methods). Separation of the translation products on SDS–polyacrylamide gels allowed the visualization of a protein with an apparent molecular mass of 58 kDa corresponding to the expected size for the VPg-Pro-N-Pol-CAT precursor (Fig. 2b). The translation was arrested by the addition of RNase A (see Materials and Methods), and the labeled precursor was incubated at 20°C to allow proteolytic processing. On incubation of the wild-type precursor, two new proteins were produced with apparent molecular masses of 30 and 28 kDa corresponding to the expected sizes for the VPg-Pro and the Pol-CAT cleaved products (Fig. 2b, lanes 1–7). Polyclonal antibodies raised against the VPg and monoclonal antibodies raised against the protease (Wang *et al.,* 1999) could immunoprecipitate the 58-kDa precursor and the 30-kDa protein but not the 28-kDa protein, confirming that the 30-kDa protein is the VPg-Pro cleaved product (data not shown). The 30- and 28-kDa proteins were not observed on incubation of the precursor containing an inactive protease (Fig. 2b, lanes 8–14), indicating that they were produced by proteolytic cleavage through the action of the endogenous protease present on the precursor. As shown previously for the VPg-Pro-N-Pol precursor, cleavage at the VPg-Pro site was not detected in the VPg-Pro-N-Pol-CAT precursor. Labeling of the 28-kDa protein was more intense than that of the 30-kDa protein due to the presence of a much larger number of methionines in the 28-kDa protein. Proteolytic processing of the wild-type precursor at the Pro-Pol site was therefore quantified by measuring the percentage of the 58-kDa precursor converted to the 28-kDa cleaved product (see Materials and Methods). Similar results were obtained by measuring the percentage of the 58-kDa precursor converted to the 30-kDa protein (data not shown). The reaction rate remained linear for ~30 min and reached a plateau after 2–4 h. After 4 h of incubation, 70–80% of the precursor was converted to the 28-kDa cleaved product (Figs. 2c and 3a). Fifty percent of this maximum conversion occurred after ~15 min (Fig. 2c).

To determine whether processing at the Pro-Pol cleavage site by the endogenous protease was predominantly *in cis* or *in trans,* a dilution experiment was conducted. The pT7VPg-Pro-N-Pol-CAT translation products were diluted in 0.1 M Tris–HCl, pH 8.0. The endogenous protease was able to process the Pro-Pol cleavage site at an equivalent rate at all the dilutions tested (1×, 10×, 100×; Fig. 3a), suggesting that proteolytic cleavage at the Pro-Pol site was predominantly an intramolecular (*in cis)* event under the conditions tested. To confirm that the Pro-Pol cleavage site could not be processed by the protease *in trans,* mutated VPg-Pro-N-Pol-CAT precursors containing inactive proteases were incubated in the presence of exogenous ToRSV protease. The source of exogenous protease was purified recombinant protease, which was shown to be active on RNA-2-encoded cleavage sites (see below). Two different precursors were tested that contained a mutation in the catalytic triad (described above) and in the substrate-binding pocket (His^{1451} mutated to Leu) (Hans and Sanfaçon, 1995). The two mutated precursors containing inactive proteases did not undergo proteolytic processing (Fig. 3b, lanes 2 and 4). This was consistent with our previous observations (Hans and Sanfaçon, 1995; Wang *et al.,* 1999). The addition of the exogenous protease to these precursors did not result in any processing of the precursors (Fig. 3b, lanes 3 and 5).

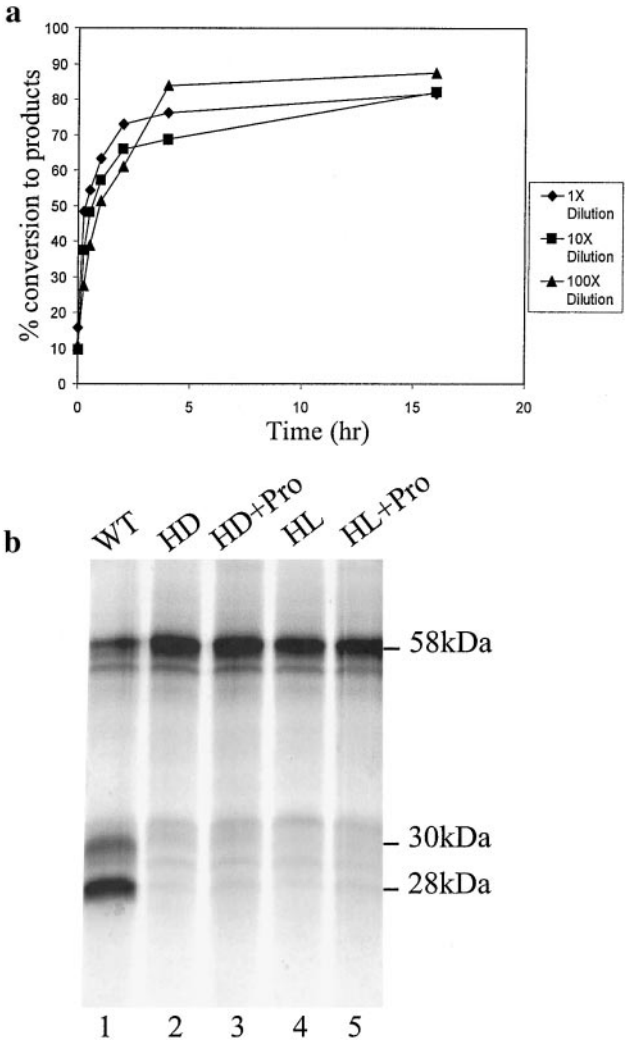


FIG. 3. Intramolecular processing at the Pro-Pol cleavage site *in vitro*. (a) Time course of processing of the VPg-Pro-N-Pol-CAT precursor to the 28-kDa product at different dilutions. After translation, the samples were diluted as indicated in 0.1 M Tris–HCl, pH 8.0. (b) Analysis of the processing of precursors containing a defective protease by an exogenous protease. VPg-Pro-N-Pol-CAT precursors containing a wild-type (WT) or mutated protease (HD and HL) were incubated for 4 h after translation in the presence (+Pro) or absence of recombinant protease purified from *E. coli* (HL indicates protease mutated in the substrate-binding pocket).

Identification and characterization of the cleavage site at the N-terminus of the movement protein

To identify the cleavage site at the N-terminus of the movement protein, cDNA clone pT3X-MP was constructed. This plasmid includes the coding region for the N-terminal portion of MP and the C-terminal portion of the protein immediately upstream of MP (protein X) under the control of the T3 RNA polymerase promoter (Fig. 4a). *In vitro* translation of run-off transcripts derived from this plasmid resulted in the production of small amounts of a protein with an estimated molecular mass of 63 kDa, which corresponded to the expected size for the X-MP precursor (Fig. 4b, lane 1). A predominant protein of ~43 kDa was also observed (Fig. 4b, lane 1). Examination of the sequence in the coding region of protein X revealed the presence of one alternate AUG codon in an optimal context for translation initiation according to the criteria established by Kozak (1987). Initiation at this codon would result in the production of a precursor containing a region of protein X with an estimated size of 5 kDa followed by the entire MP region (estimated size, 38 kDa) (Fig. 4a). Immunoprecipitation experiments with polyclonal antibodies raised against the C-terminal 31 kDa of MP (Fig. 4a) confirmed that both the 43- and 63-kDa proteins contained this region of MP (data not shown).

To test for the presence of the X-MP cleavage site in the 63- and 43-kDa precursors, exogenous ToRSV protease was added to the translation products of plasmid pT3X-MP. The source of protease was either purified recombinant ToRSV protease or cold translation products obtained from plasmids pT7VPg-Pro-N-Pol-II or pT7VPg-Pro-N-Pol-CAT. On the addition of either protease, the amount of the predominant 43-kDa precursor decreased over time, whereas a new protein with an estimated molecular mass of 38 kDa, which corresponded to the expected size for the MP cleaved product, was produced (Fig. 4b). The nature of the 38-kDa protein was confirmed by immunoprecipitation with polyclonal antibodies raised against the C-terminal half of the movement protein (data not shown) and by microsequencing its N-terminus (see below). The large amount of the 38-kDa protein produced indicated that it was released mainly from the 43-kDa precursor. An additional protein with an estimated molecular mass of 25 kDa was also occasionally detected in very low amounts (Fig. 5a, lane 2). The estimated size of this protein corresponded to the expected size for the cleaved product of the X protein (Fig. 4a). This protein was predicted to be produced from the 63-kDa precursor but not from the 43-kDa precursor; therefore, cleavage of the 63-kDa precursor was likely to contribute to a small extent to the production of the 38-kDa product. For quantification of the processing efficiency, the percentage of conversion of the 63- and 43-kDa precursors to the 38-kDa product was calculated as described in Materials and Methods. Very similar results were obtained when only the 43-kDa precursor was included in the calculation (data not shown). As expected from a cleavage site recognized *in trans*, the efficiency of proteolytic processing was dependent on the dilution of the exogenously supplied protease (data not shown). To allow meaningful comparison of cleavage at the Pro-Pol and X-MP sites, the concentration of protease added to the X-MP precursor was adjusted to obtain kinetics similar to those observed for the *cis* cleavage in the VPg-Pro-N-Pol-CAT precursor (Fig. 4c). Under those conditions, the reaction rate remained linear for ~30 min with a maximum of conversion of the precursors to the product of ~85%. Similar to the VPg-Pro-N-Pol-CAT cleavage kinetics, half of this maximum conversion occurred after ~15 min (Fig. 4c).

On examination of the amino acid sequence in the region N-terminal of the movement protein, two potential cleavage sites were predicted: Q^{907}/S and Q^{934}/S. Mutants of pT3X-MP were obtained in which the glutamine was deleted from each of these potential cleavage sites (pT3X-MPΔQ^{907} and pT3X-MPΔQ^{934}). When protease purified from *Escherichia coli* was added to the pT3X-MPΔQ^{907} mutant translation products, processing of the 63- and 43-kDa precursors into the 25- and 38-kDa cleaved products was observed (Fig. 5a, lanes 3 and 4). However, when the purified protease was added to the pT3X-MPΔQ^{934} mutant translation products, processing of the 63- or 43-kDa precursors was not detected (Fig. 5a, lanes 5 and 6). This suggested that Q^{934}/S was the X-MP cleavage site. To further confirm the nature of the cleavage site, translation of the wild-type clone pT3X-MP was performed in the presence of [^{3}H]leucine and then recombinant protease was added and the 38-kDa cleaved product was eluted from the SDS–polyacrylamide gel and subjected to Edman degradation. The presence of two peaks of radioactivity at amino acid positions 3 and 8 is consistent with S^{935} being the first amino acid of the 38-kDa cleaved product (Fig. 5b).

Site-directed mutagenesis of the Pro-Pol cleavage site

Comparison of ToRSV cleavage sites suggest that amino acids at the −2, −1, and +1 positions are conserved (see Fig. 1). Furthermore, amino acids in the −6 to +1 positions are conserved in the cleavage sites of many related viruses (see Fig. 1). To determine whether amino acids in the vicinity of the X-MP and Pro-Pol cleavage sites were required to define the ToRSV cleavage sites, amino acid substitutions in the −6 to +1 positions were introduced into the VPg-Pro-N-Pol-CAT and X-MP precursors. The substitutions tested were either conservative changes (e.g., Ser to Thr) or drastic changes (e.g., Ser to Phe). Ala was also introduced in each position. In addition, specific amino acids were introduced that were found in cleavage sites from other

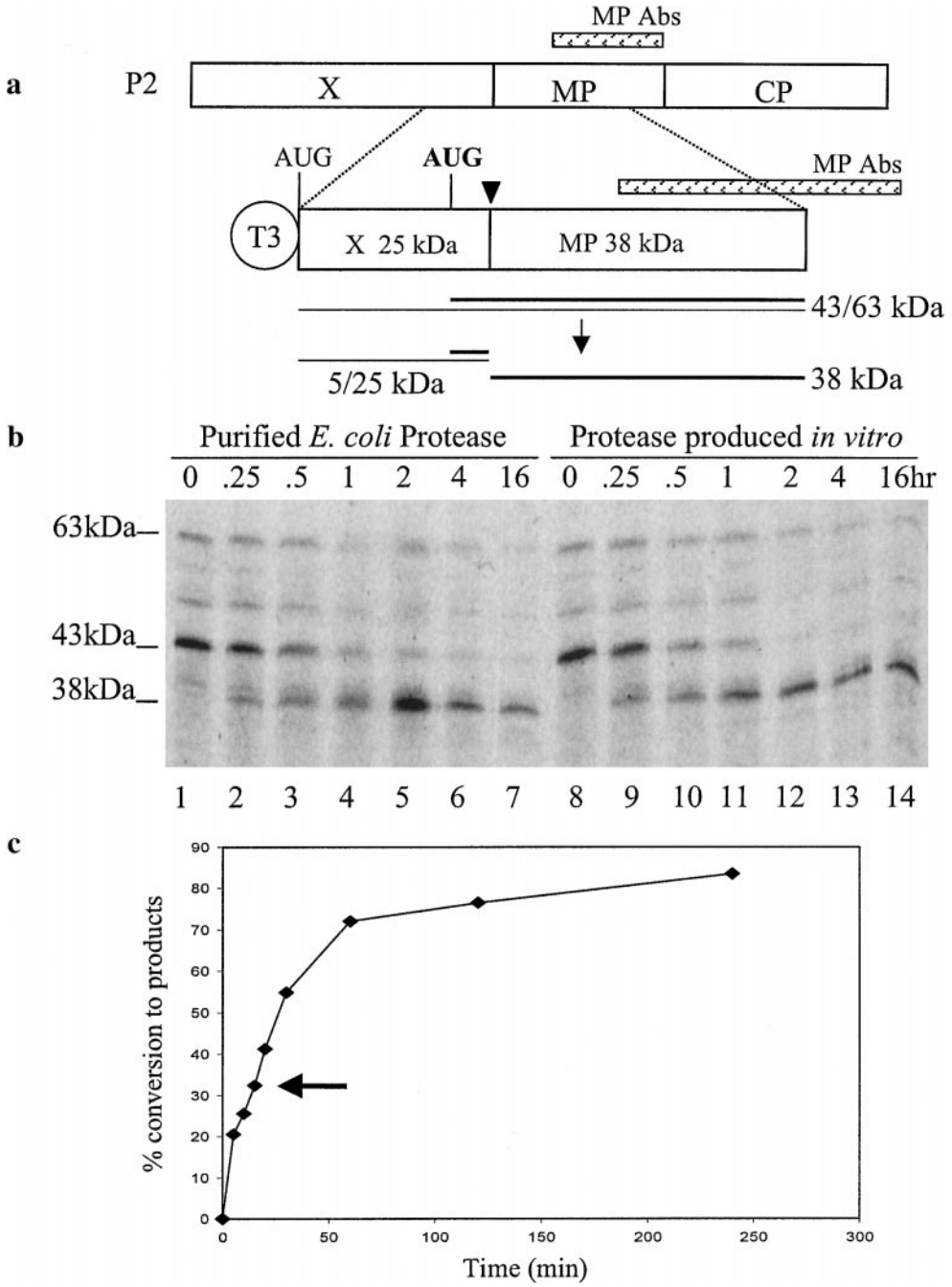


FIG. 4. Construction of the X-MP precursor and proteolytic processing at the X-MP cleavage site *in vitro*. (a) Schematic representation of the cDNA clone encoding the X-MP precursor. The entire P2 polyprotein is shown at the top of the figure and plasmid PT3X-MP is shown below. The coding regions for the different ToRSV protein domains are shown along with the predicted molecular mass of each domain (X indicates protein of unknown function). The X-MP cleavage site is indicated by the arrowhead. Two AUG codons are shown, the second of which may be responsible for the production of the 43-kDa precursor through internal initiation. The rectangles shown above the MP domains in the P2 and X-MP precursors represent the region of MP against which polyclonal antibodies were raised (MP Abs). The predicted translation products are shown before (63- and 43-kDa precursor) and after (38-, 25-, and 5-kDa cleaved products) processing by the exogenously supplied ToRSV protease. (b) Processing of the X-MP precursor *in vitro*. Processing was tested using ToRSV protease purified from *E. coli* (lanes 1–7) or produced *in vitro* (lanes 8–14). The translation products were separated on 12% SDS–polyacrylamide gels. The time of incubation of the different samples is indicated above each lane (hr indicates hours). The expected position for the precursors and cleaved products is indicated on the left side of the gel. (c) Time course of processing of the X-MP precursor to the 38-kDa product by recombinant protease purified from *E. coli*. The arrow indicates the percentage of conversion at the 15-min time point.

nepoviruses, from comoviruses or from picornaviruses (e.g., Glu, Asn, Lys, and Arg in the −1 position and Gly and Met in the +1 position) (Fig. 1). Time course experiments were performed on each mutant as described above. Kinetic analysis of each mutant was performed in parallel with the corresponding wild-type precursor at

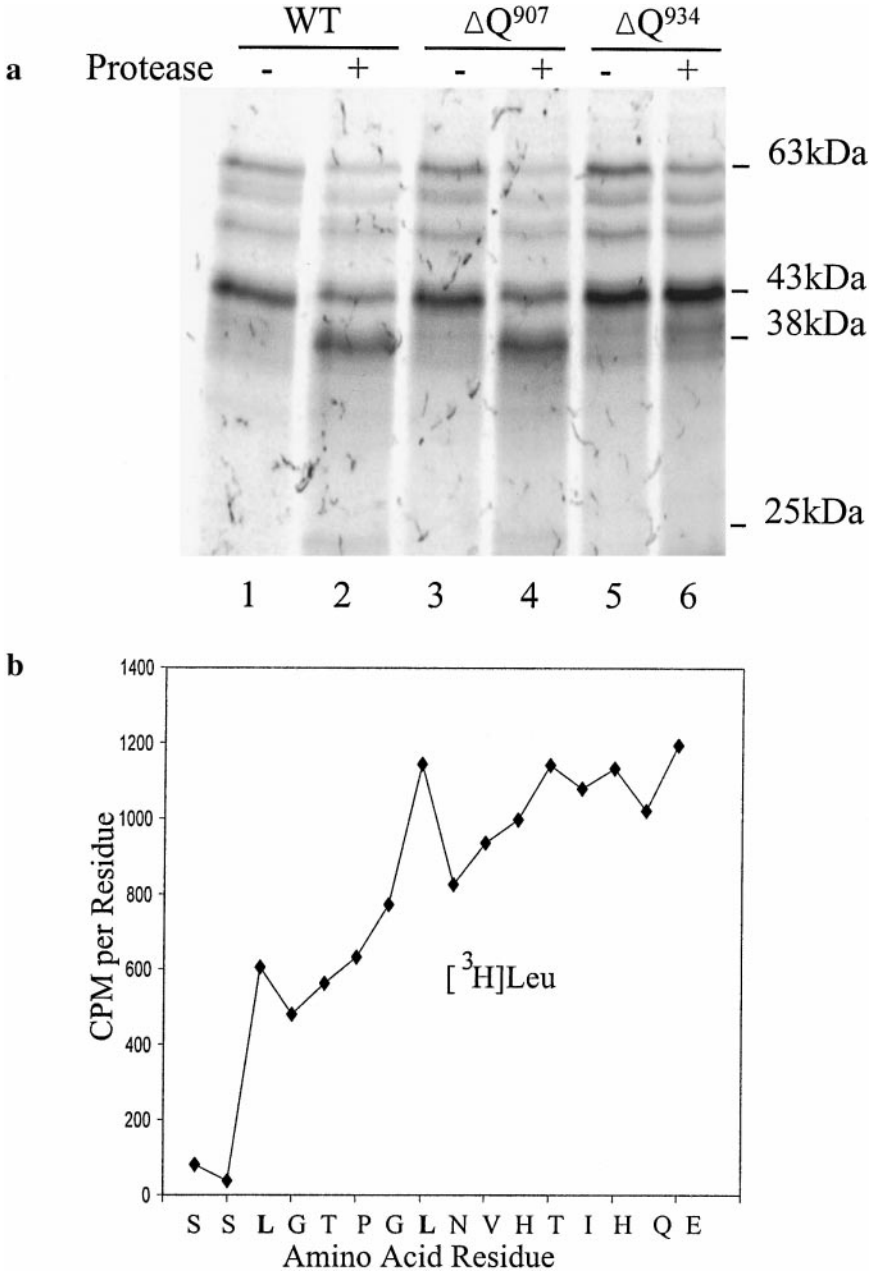
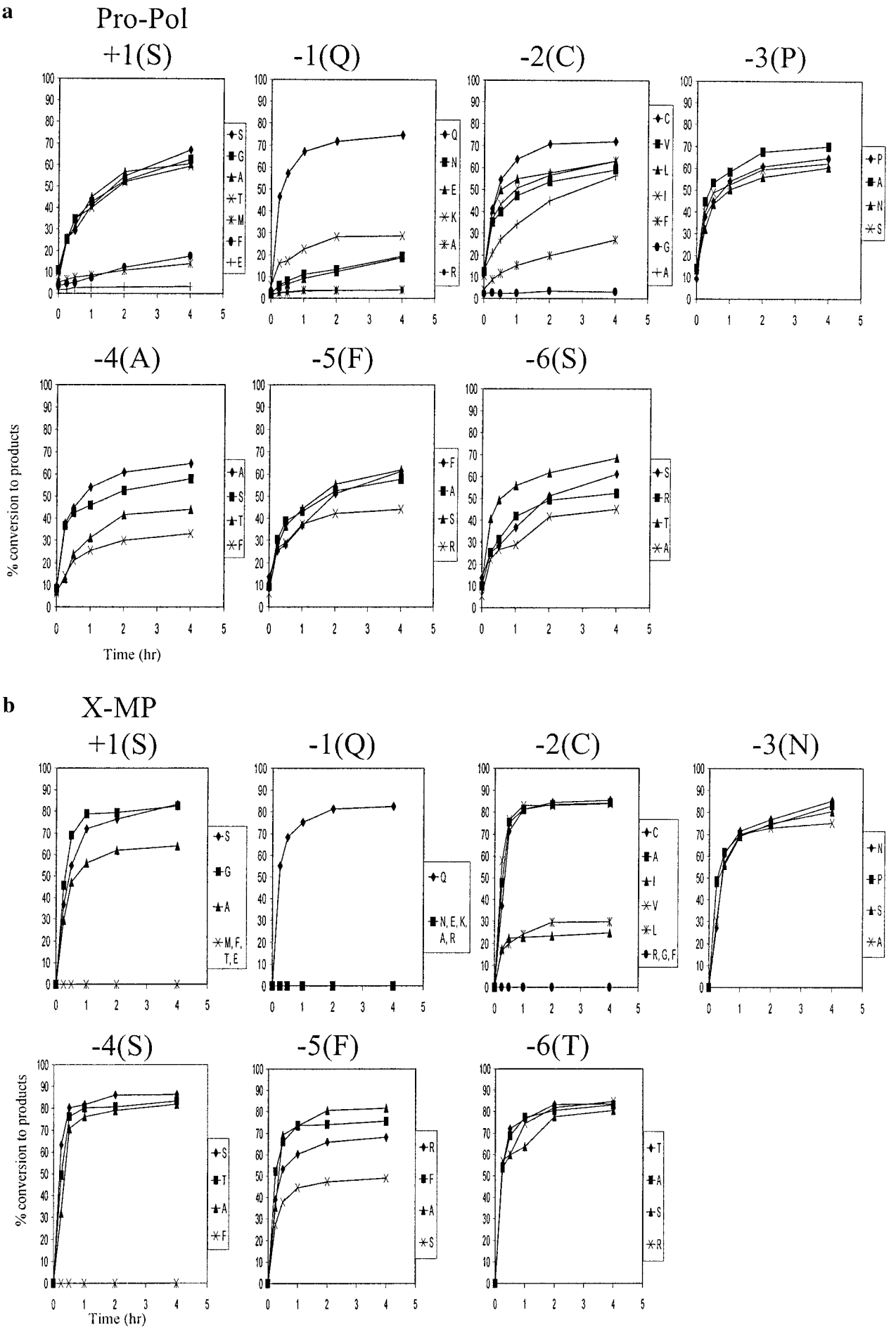


FIG. 5. Identification of the X-MP cleavage site. (a) Effect of mutations of predicted cleavage sites on proteolytic processing *in vitro*. Translation products from the wild-type X-MP precursor (WT) or from two mutant derivatives, including a deletion of the Gln at the −1 position in the predicted Q^{907}/S (ΔQ^{907}) and Q^{934}/S (ΔQ^{934}) cleavage sites, were incubated in the presence (+) or absence (−) of purified recombinant protease. The translation products were separated using 12% SDS–PAGE. (b) N-terminal microsequencing of the [^{3}H]leucine-labeled 38-kDa cleaved product released from the maturation of the wild-type X-MP precursor with ToRSV protease. The amount of radioactivity measured in the fractions collected at each cycle of the Edman degradation procedure is shown in cpm. The amino acid sequence shown below the graphic corresponds to the amino acid sequence of the ToRSV polyprotein starting at S^{935} located immediately downstream of the putative Q^{934}/S cleavage site.

least three times, and a representative experiment is shown. Processing efficiency of the mutated cleavage sites was compared with that of the wild-type at the 15-min time point. As mentioned above, at this time, the rates of processing of the wild-type X-MP and VPg-Pro-N-Pol-CAT precursors at the X-MP and Pro-Pol cleavage sites were linear.

Kinetics of processing of the Pro-Pol cleavage site for each individual mutant are shown in Fig. 6a. Relative efficiency of processing of the mutant cleavage sites compared with that of the wild-type cleavage site at the 15-min time point is shown in Fig. 7a. Substitution of Ser at the +1 position of the Pro-Pol cleavage site had a variable effect on processing efficiency.


a
Pro-Pol
+1(S)
-1(Q)
-2(C)
-3(P)
-4(A)
-5(F)
-6(S)
% conversion to products
Time (hr)
b
X-MP
+1(S)
-1(Q)
-2(C)
-3(N)
-4(S)
-5(F)
-6(T)
% conversion to products
Time (hr)

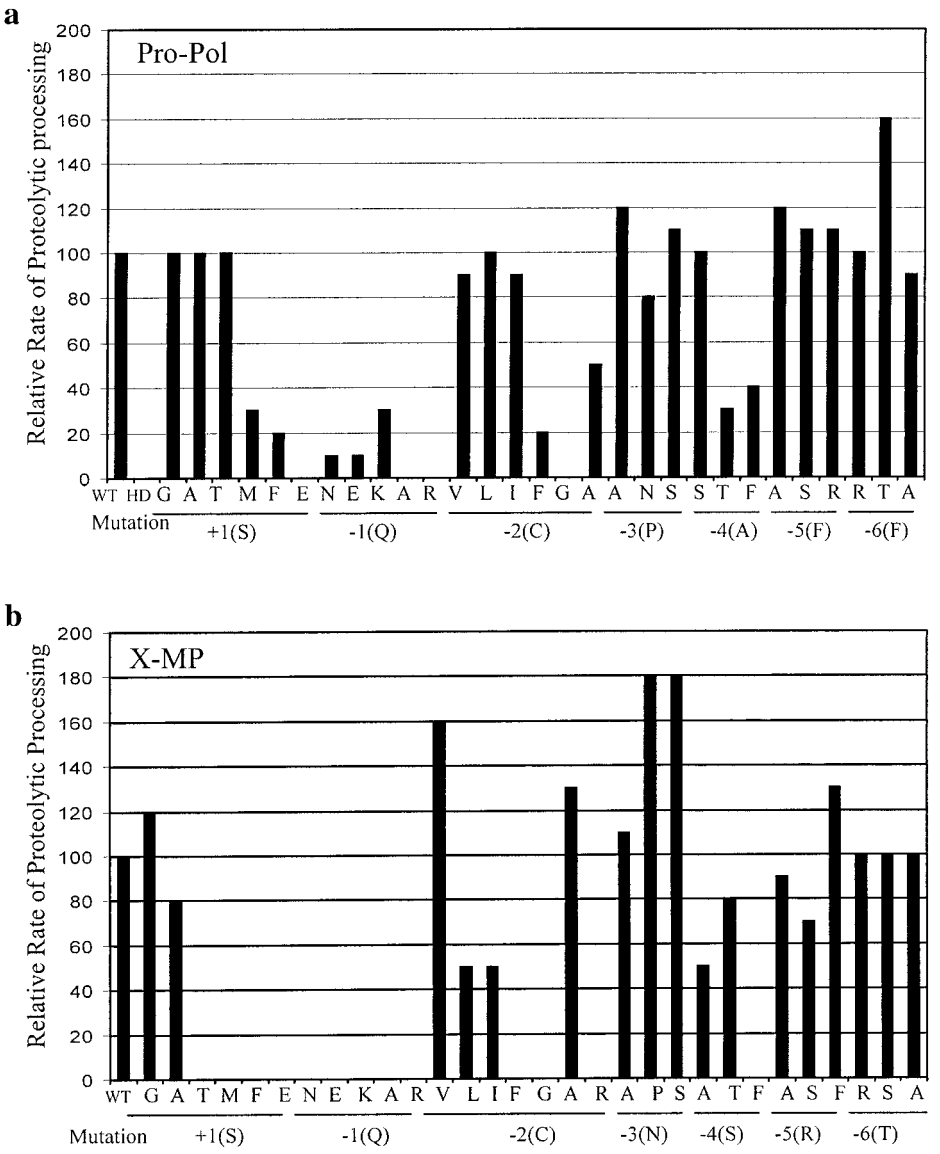


FIG. 7. Relative processing efficiency of mutated ToRSV cleavage sites at the 15-min time point. (a) Comparative analysis of *cis* processing of Pro-Pol cleavage site mutations by the ToRSV protease. Relative processing efficiency at the 15-min time point, expressed as a percentage of the processing efficiency of the wild-type precursor, is shown for each mutant. (b) Comparative analysis of *trans* processing of X-MP cleavage site mutations by the ToRSV protease. Processing efficiency is expressed as in a.

Substitutions with Gly, Ala, or Thr did not appear to influence the kinetics of proteolytic processing. In contrast, substitution with Met, Phe, or Glu resulted in a significant reduction in proteolytic processing. After a 15-min incubation, processing of precursors with an introduced Met and Phe at the +1 position was ~30% and 20% that of the wild-type precursor, respectively. Very little processing (if any) was detected for the Glu mutant. Mutation of Gln at the −1 position by substituting any of the amino acids tested (Asn, Glu, Lys, Ala, and Arg) resulted in a significant reduction in proteolytic processing. After a 15-min incubation, processing efficiency was ~30%, 10%, and 10% of that of the wild-type for the Lys, Glu, and Asn mutants, respectively. Very little processing (if any) was detected for the Ala and Arg mutants. Substitution of Cys at the −2

FIG. 6. Kinetics of proteolytic processing at mutated ToRSV cleavage sites. (a) Effect of mutations on the proteolytic processing of the Pro-Pol cleavage site *in vitro*. Time course of the conversion of VPg-Pro-N-Pol-CAT precursor to the 28-kDa product is shown. (b) Effect of mutations on the proteolytic processing of the X-MP cleavage site. Time course of the conversion of the 63- and 43-kDa precursors to the 38-kDa product is shown.

position had a range of effects on proteolytic processing that depended on the nature of the amino acid introduced. Substitutions with the aliphatic amino acids Val, Leu, or Ile resulted in kinetics that showed little or no reduction in cleavage efficiency compared with the wild type. Substitution with Ala and Phe resulted in kinetics that showed a lower level of proteolytic processing than with the other aliphatic amino acids. After a 15-min incubation, processing efficiency of precursors containing a substitution with Ala and Phe was ~50% and 20% that of the wild-type precursor, respectively. Processing was very inefficient (or absent) in the mutated cleavage site in which Gly was introduced at the −2 position. Amino acid substitution of Ala at the −4 position did show some effect on processing efficiency depending on the specific mutant tested. Replacement of the Ala with Ser resulted in kinetics that were very similar to that of the wild type. However, when Thr or Phe was introduced at that position, cleavage at the Pro-Pol site was less efficient. After 15 min, relative processing efficiency of the Thr and Phe mutants was 30–40% of that of the wild type (Fig. 4c). Introduced changes in the −3 and −5 positions did not seem to have much effect on the ability of the protease to process the Pro-Pol cleavage site *in vitro*. Substitution of Pro at the −3 position with Ala, Asp, and Ser resulted in processing kinetics that were similar to those of the wild-type cleavage site. Similarly, replacement of the Phe at the −5 position by Ala, Ser, or Arg did not seem to affect the efficiency of processing. Mutation of the Ser at the −6 position did not have a negative effect on the ability of the protease to process the Pro-Pol cleavage site *in vitro*. The introduction of Arg or Ala in this position resulted in kinetics that were similar to those of the wild type (Fig. 4a). Substitution with Thr resulted in kinetics that showed a level of proteolytic processing that was higher than that of the wild type. Indeed, processing efficiency was ~160% that of the wild-type precursor after 15 min.

The VPg-Pro-N-Pol-CAT precursor used in the above experiments included an in-frame fusion to the CAT protein. To verify that the presence of this protein on the precursor did not alter recognition of the cleavage sites, several of the amino acid substitutions discussed above were introduced in the cDNA clone pT7VPg-Pro-N-Pol-II. Results from time course experiments of these mutated precursors were similar to those obtained for the corresponding mutated VPg-Pro-N-Pol-CAT precursor (data not shown). Finally, to verify that substitution of amino acids at the Pro-Pol cleavage site did not affect the catalytic activity of the protease itself, several mutants were tested for their ability to cleave the X-MP cleavage site *in trans*. Cold translations of amino acid substitutions at the −1 position, which resulted in loss of cleavage at the Pro-Pol site, were incubated in the presence of the X-MP precursor. Processing at the X-MP cleavage site was comparable to that obtained from incubation of the X-MP precursor with the cold translation products from the wild-type pT7VPg-Pro-N-Pol-CAT plasmid (data not shown). Taken together, these results suggested that amino acids at positions −2, −1, and +1 and possibly −4 and −6 played an important role in the efficiency of processing of the Pro-Pol cleavage site by the ToRSV protease.

Site-directed mutagenesis of the X-MP cleavage site

The effects of point mutations on the X-MP cleavage site are shown in Figs. 6b and 7b, as described above. The results shown were obtained from incubation of the pT3X-MP precursor containing mutated cleavage sites with purified recombinant ToRSV protease. Similar results were observed from incubation of several representative mutants with ToRSV protease produced in the rabbit reticulocyte system (cold *in vitro* translation products of wild-type pT7VPg-Pro-N-Pol-II or pT7VPg-Pro-N-Pol-CAT plasmids; data not shown).

Introduced changes at the +1 position had various effects on the ability of the protease to process the X-MP cleavage site *in vitro*. Only Ser (the wild-type amino acid), Gly, or Ala was tolerated at this position. After a 15-min incubation, processing efficiency was ~120% and 80% that of the wild-type for the Gly and Ala mutants, respectively. Processing was not detected in mutants that included a Met, Phe, Thr, or Glu at the +1 position. Replacement of Gln at the −1 position by any of the amino acids tested (Asn, Glu, Lys, Ala, or Arg) resulted in undetectable processing at the cleavage site. Several amino acid substitutions at the −2 position showed a dramatic effect on processing efficiency. Of the amino acids tested, only Cys (wild type), Ala, or Val resulted in efficient processing at the X-MP site. After a 15-min incubation, processing efficiency was ~130% and 160% that of the wild-type for the Ala and Val mutants, respectively. The introduction of Ile or Leu resulted in decreased cleavage at the X-MP site. After 15 min, relative processing efficiency compared with the wild type was ~50% for the Ile and Leu mutants. Replacement of Cys by other amino acids (Arg, Phe, or Gly) prevented processing at the cleavage sites. The effect of mutations at the −4 position varied with the specific amino acid introduced. Replacement of Ser at the −4 position with Thr or Ala resulted in reduced processing efficiency. After 15 min, processing efficiency was ~80% and 50% of the wild type for the Thr and Ala mutant, respectively. The introduction of Phe at this position resulted in a drastic reduction of proteolytic processing (processing was not detectable). Amino acid substitutions in the −3, −5, and −6 positions did not have a dramatic effect on the ability of the protease to process the X-MP cleavage site *in vitro*. The substitution of Asp at the −3 position with Pro,

Ser, or Ala resulted in kinetics that showed similar levels of proteolytic processing to those of the wild-type cleavage site. The substitution of Arg at the −5 position with Phe or Ala only slightly influenced processing efficiency. The introduction of Ser at this position resulted in a small reduction in proteolytic processing (processing efficiency was ~70% that of the wild type after 15 min). Replacement of Thr at the −6 position with Ala, Ser, or Arg did not influence the efficiency of processing at the cleavage site. Taken together, these results suggest that amino acids at positions −2, −1, +1, and possibly −4 play a critical role in the ability of the protease to cleave the X-MP cleavage site.

DISCUSSION

The results of the site-directed mutagenesis experiments presented in this study provide direct evidence for the importance of conserved amino acids at the −2, −1, and +1 positions of ToRSV cleavage sites. Indeed, in both cleavage sites analyzed, mutations introduced at these positions were found to have the most pronounced effect on cleavage efficiency. Several features of the ToRSV cleavage sites defined here were also found in those of the related picornaviruses and potyviruses. Previous studies on TEV and HAV cleavage sites have shown that similar to the ToRSV cleavage sites, the −1 position was the most sensitive to amino acid substitutions (Dougherty *et al.,* 1988; Jewell *et al.,* 1992). In ToRSV, the substitution of Gln at the −1 position with Glu or Asn allowed some processing of the Pro-Pol cleavage site. Interestingly, these amino acids are present in the −1 position of other picorna-like cleavage sites: Glu is found in picornavirus cleavage sites (rhinovirus and HAV), and Asn is found in one subgroup c nepovirus cleavage site (blueberry leaf mottle virus) (Fig. 1). Although requirements for specific amino acids were less stringent at the +1 position, substitution with Met resulted in a dramatic decrease of proteolytic processing for both ToRSV cleavage sites. This amino acid is present in the cleavage sites of a comovirus (CPMV) (Wellink *et al.,* 1986). These results suggest that the ToRSV protease prefers a small amino acid, such as Gly, Ser, or Ala, at the +1 position, whereas the CPMV protease can accommodate a larger residue at this position.

In ToRSV, the −2 position was defined as an important determinant of the X-MP and Pro-Pol cleavage sites. The protease had a preference for Cys or for an aliphatic amino acid, such as Val or Ala, at this position. A Cys or Val is found at the −2 position in cleavage sites of other subgroup c nepoviruses and of some subgroup a and b nepoviruses, whereas a conserved Ala or Pro is found the −2 position of CPMV (Fig. 1). Conserved amino acids at the −2 position were also found in turnip mosaic virus (a potyvirus) (Fig. 1). Therefore, requirements for specific amino acids at the −2 position may not be unique to the ToRSV cleavage sites. In contrast, in TEV, HAV, and poliovirus, specific amino acids were not conserved at the −2 position (Dougherty *et al.,* 1988; Blair and Semler, 1991), and substitutions at the −2 position of the HAV cleavage site did not alter processing efficiency (Jewell *et al.,* 1992). The cleavage sites of picornaviruses, potyviruses, and comoviruses contain a small aliphatic amino acid at the −4 position (Fig. 1), which is critical in defining a functional cleavage site in poliovirus, human rhinovirus, and TEV (Pallai *et al.,* 1989; Cordingley *et al.,* 1990; Blair and Semler, 1991; Dougherty *et al.,* 1988). In contrast, ToRSV cleavage sites did not contain a conserved amino acid in the −4 position, and mutagenesis of this position resulted in modest changes in the processing efficiency, with the exception of substitution of the Ser at the −4 position of the X-MP cleavage site with Phe, which resulted in a loss of proteolytic processing. This loss of processing might have been the result of a severe conformation change. Although only a limited number of mutations have been tested at the −4 position, our results suggest that the presence of specific amino acids at the −4 position may modulate processing efficiency at the cleavage site but may not be a primary determinant in the definition of the cleavage sites.

We have shown that the presence of a Q/(G or S) dipeptide combined with a Cys or Val at the −2 position are important substrate determinants for the ToRSV protease. However, at least one cleavage site that met these requirements was not cleaved by the protease. In our *in vitro* assay, tripeptide VQ^{907}/S present on the X-MP precursor was not recognized by the ToRSV protease either in a wild-type precursor or in a precursor in which the Q^{934}/S cleavage site was deleted. This suggests that other factors, such as secondary structures, are likely to be important for the definition of ToRSV cleavage sites, as shown by Ypma-Wong *et al.* (1988) for the poliovirus cleavage sites. Although processing at the Q^{907}/S dipeptide was not detected *in vitro*, we cannot exclude the possibility that it may be processed *in vivo* in the entire P2 polyprotein, which may present a different secondary structure around this site. This may provide an explanation for our previous observation that two closely migrating species of the movement protein are detected on SDS–PAGE analysis of extracts of ToRSV-infected protoplasts (Sanfaçon *et al.,* 1995).

Under the experimental conditions used, processing at the Pro-Pol cleavage site was predominantly an intramolecular event *in vitro*. Similarly, the Pro-Pol cleavage sites of several related viruses are also cleaved *in cis* (Palmenberg and Rueckert, 1982; Hanecak *et al.,* 1984; Hemmer *et al.,* 1995; Margis *et al.,* 1994; Carrington and Dougherty, 1987). In poliovirus, although the cleavage is predominantly a *cis* event early in infection when the concentration of the protease is low, it can become a *trans* event late in infection (Palmenberg and Rueckert,

1982; Hanecak *et al.,* 1984). Although we could not detect *trans*-cleavage *in vitro*, we cannot exclude the possibility that it could occur at higher concentrations of the protease. In addition, *trans* cleavage may be observed in larger protease precursors not tested in this study.

The results presented here show that amino acid substitutions were more tolerated at the Pro-Pol cleavage site than at the X-MP cleavage site. This was most evident in the −1 position. In the X-MP cleavage site, any substitution appeared to eliminate proteolytic processing, whereas several substitutions resulted in some cleavage at the Pro-Pol site. These results raise the possibility that the requirements for specific amino acids are more stringent at cleavage sites recognized *in trans* than *in cis*. Analysis of other cleavage sites would be necessary to confirm this suggestion. Similar to our results, the flavivirus NS3 protease (Bartenschlager *et al.,* 1995) and the poliovirus 2A protease (Hellen *et al.,* 1992) were reported to have more stringent requirements for specific amino acids at *trans*-cleavage sites rather than at *cis*-cleavage sites. To our knowledge, substrate determinants for *cis* and *trans* cleavage have not been systematically compared for other 3C-like proteases. In CPMV, mutation of the Gln/Ser dipeptide to His/Met at a *cis*-cleavage site resulted in efficient cleavage (Peters *et al.,* 1992). In contrast, mutation of the +1 position in a *trans*-cleavage site resulted in drastic reduction of processing (Vos *et al.,* 1988). Although the effects of equivalent mutations were not compared in both cleavage sites, these results suggest that similar to our results, the requirement for specific amino acids are less stringent at a *cis*-cleavage site than at a *trans*-cleavage site in CPMV (Goldbach and Wellink, 1996).

The results presented in this study are useful for establishing the cleavage site specificity of the ToRSV protease; however, there are limitations in the system used. First, cleavage sites were characterized in partial polyproteins rather than in the entire RNA1- or RNA2-encoded polyproteins. Second, we only looked at the rates of proteolytic processing *in vitro*. Despite these limitations, it is noteworthy that the requirements for specific amino acids at the −2, −1, and +1 position were similar in the two ToRSV cleavage sites studied. Definition of the effect of cleavage site mutations on virus amplification *in vivo* will await the construction of infectious transcripts.

MATERIALS AND METHODS

Plasmid constructions

To construct plasmid pT3X-MP, the 1674-bp *Eco*RV-*Mlu*I fragment (ToRSV RNA2 nucleotides 2258–3932) from pMR14 (Rott *et al.,* 1991) was ligated into the *Sma*I–*Mlu*I sites of the polylinker of plasmid pMTL23 (Chambers *et al.,* 1988). This allowed an in-frame fusion of the ToRSV-derived reading frame with an AUG codon present on the *Nco*I site of the polylinker from plasmid pMTL23. A *Xho*I–*Eco*RV fragment was obtained from this intermediate plasmid and ligated into the corresponding sites of plasmid pKS(+) (Stratagene, La Jolla, CA).

To construct plasmids pT7VPg-Pro-N-Pol-CAT, pT7VPg-Pro^{H1283D}-N-Pol-CAT, and pT7VPg-Pro^{H1451L}-N-Pol-CAT, a fragment containing the CAT coding region was amplified from plasmid pCaMVCN (Pharmacia) using oligonucleotides KC-53 (5′-TTGAAAAGCTTGAGAAA-AAAATCACTGGATAT-3′, *Hin*dIII site underlined) and KC-54 (5′-TATATCTCGAGAAATTACGCCCCGCCC-3′, *Xho*I site underlined) and *Pfu* polymerase (Stratagene). This fragment was digested with *Hin*dIII and *Xho*I and ligated into the corresponding sites of plasmids pT7VPg-Pro-N-Pol-II, pT7VPg-Pro^{H1283D}-N-Pol-II (Wang *et al.,* 1999), and pT7VPg-Pro^{H1451L}-N-Pol-II. Plasmid pT7VPg-Pro^{H1451L}-N-Pol-II was constructed by ligating the 417-nucleotide *Bam*HI–*Eco*RI fragment from clone pMR10 (ToRSV RNA1 nucleotides 4436–4852) with the large *Bam*HI–*Eco*RI fragment of plasmid pT7Pro^{H1451L} (Hans and Sanfaçon, 1995).

Plasmid pETMP was created to allow the production of the MP-His fusion protein containing the C-terminal part of the movement protein in *E. coli.* This protein was used as an antigen for the preparation of polyclonal antibodies against the movement protein (see below). Plasmid pET-MPCAT was first constructed by inserting the *Sac*I–*Sal*I fragment of pT7MPCAT (Hans and Sanfaçon, 1995) into the *Sac*I–*Xho*I sites of the polylinker from plasmid pET21b (Novagen, Madison, WI). Plasmid pETMP was then obtained by subcloning the 792-nucleotide *Eco*RI fragment from plasmid pET-MPCAT into the *Eco*RI site of the polylinker from plasmid pET21b.

Site-directed mutagenesis

Mutations of the Pro-Pol and X-MP cleavage sites were introduced by mutating the corresponding codon in the cDNA clones pT3X-MP and pT7VPg-Pro-N-Pol-CAT, using the Quickchange site-directed mutagenesis kit (PDI Bioscience). Mutagenic primers, corresponding to the region of the ToRSV cleavage site, were 27–32 nucleotides in length and contained one- or two-point mutations in the middle of the sequence. The presence of the mutations was verified by DNA sequencing. Oligonucleotides KC-9 (5′-CGTCTGCCCCAGTACAGT-3′) and KC-89 (5′-GAAGGTGGTTACAAAATCATA-3′) were used as sequencing primers for the pT3X-MP and pT7VPg-Pro-N-Pol-CAT cleavage site mutants, respectively. The sequencing primers bound to a region ~150 nucleotides upstream of the cleavage site.

In vitro translation and proteolytic processing

In vitro expression of the wild-type and mutated pT7VPg-Pro-N-Pol-CAT cDNA clones was performed using a TNT coupled transcription/translation system (Pro-

mega, Madison, WI). Translation products were labeled with [^{35}S]methionine for 30 min at 30°C. Translation was arrested by adding 5 units of RNase A (Promega). The translation products were further incubated at 20°C in the presence of 0.1 M Tris–HCl, pH 8.0, to allow proteolytic processing. Reactions were stopped by the addition of 2× protein sample buffer (Laemmli, 1970). For protease dilution experiments, the translation products were diluted using 0.1 M Tris–HCl, pH 8.0.

Run-off transcripts were synthesized from linearized pT3X-MP plasmids using bacteriophage T3 RNA polymerase (GIBCO BRL) essentially as described previously (Hans and Sanfaçon,1995). *In vitro* translation of these transcripts was performed using a rabbit reticulocyte lysate system (Promega) as described previously (Hans and Sanfaçon, 1995). The reactions were stopped by the addition of 5 units of RNase A (Promega). To allow processing of the translation products, exogenous protease was added to the translation mixture. The source of added protease was either cold translation products of clones pT7VPg-Pro-N-PoI-II or pT7VPg-Pro-N-PoI-CAT (translations were performed as described above but in the presence of cold methionine) or recombinant protease purified from *E. coli* (see below). Proteolytic processing was allowed to proceed by incubation at 20°C and then stopped by the addition of 2× protein sample buffer.

Recombinant active protease was purified from the expression products of plasmid pET15bVPgPro-N-PoI essentially as described previously (Wang *et al.,* 1999). On expression, the protease was insoluble. Solubilization of the protease from the purified inclusion bodies with urea and renaturation of the protease by gradual dialysis were performed as described previously (Wang *et al.,* 1999). Purified protease was stored in 50 mM Tris–HCl, pH 8, 1 mM DTT, and 10% glycerol at −70°C.

Quantification of the processing reaction

To measure the amount of proteolytic processing at the wild-type and mutant cleavage sites, time course experiments were conducted. The precursors and cleaved products were separated using SDS–PAGE (Laemmli, 1970), and the gels were scanned using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The amount of radioactivity in individual bands was measured using ImageQuant. The cpm values were adjusted for the number of methionines present in each protein, and the percentage of precursor that was converted to cleaved product was calculated at each time point. This percentage was calculated based on radioactivity measured in the 58-kDa precursor and the 28-kDa cleaved product for pT7VPg-Pro-N-PoI-CAT and the 63- and 43-kDa precursors and the 38-kDa cleaved product for pT3X-MP.

N-terminal microsequencing of radiolabeled peptides

For microsequencing of the 38-kDa cleaved product resulting from the processing of the 63- and 43-kDa precursor products, pT3X-MP transcripts were translated *in vitro* in the presence of [^{3}H]leucine and incubated with purified recombinant protease as described above. The translation products were separated using SDS–PAGE. The 38-kDa protein was eluted and subjected to microsequencing as described previously (Wang *et al.,* 1999).

Production of polyclonal antibodies against the C-terminal part of the movement protein and immunoprecipitation experiments

Polyclonal antibodies were raised against a fusion protein (MP-His) consisting of the C-terminal 31 kDa of MP followed by six histidines. The MP-His fusion protein was purified from the expression products of plasmid pET-MP essentially as described above for the recombinant protease. One additional step of purification on nickel column was performed before protein renaturation as described by the supplier (Novagen); 1 mg of this purified protein was used to inject rabbits intramuscularly as described previously (Sanfaçon *et al.,* 1995). Immunoprecipitations of [^{35}S]methionine-labeled translation products were performed as described previously (Hans and Sanfaçon, 1995).

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