



# The mature reverse transcriptase molecules in virions of mouse mammary tumor virus possess protease-derived sequences

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Received 16 August 2002; returned to author for revision 20 September 2002; accepted 17 December 2002

## Abstract

Our efforts to express in bacteria the enzymatically active reverse transcriptase (RT) of mouse mammary tumor virus (MMTV) have shown that the RT is active only after adding 27 amino acid residues, which are derived from the end of the *pro* gene, to the amino-terminus of the RT (Biochem. J. (1998) 329, 579–587). In the present study we have tested whether the mature RT found in virions is also fused to protease-derived sequences. To this end, we have analyzed the RT molecules in virions of MMTV by using two antisera directed against peptides, derived from either the carboxyl-terminus of MMTV protease or the middle of MMTV RT. The data suggest that the mature RT, located in virions, contains at its amino-terminus sequences from the carboxyl-terminus of the protease protein. This finding supports previous suggestions that MMTV RT is a transframe protein (derived from both *pro* and *pol* reading frames of MMTV) and that amino acid residues located at the carboxyl-terminus of the protease have a dual usage as integral parts of both the protease and the RT enzymes.

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**Keywords:** Reverse transcriptase; Protease; Mouse mammary tumor virus; Transframe proteins; Frameshifting

## Introduction

Full-length transcripts of the integrated provirus serve as the messenger RNA (mRNA) for the synthesis of the Gag, Gag-Pro, and Gag-Pro-Pol polyprotein precursors of retroviruses (Coffin, 1996; Coffin et al., 1997). The translation of the proteins encoded by the *pro-pol* genes is performed after bypassing the termination codon at 3'-end of the *gag*-reading frame. The mechanism used in most retroviruses involves a ribosomal slippage backward by one nucleotide (a  $-1$  frameshift toward the 5'-end) during mRNA translation. Thus, the ribosomes move from the *gag* reading frame into the *pro*-reading frame. In many retroviruses, including mouse mammary tumor virus (MMTV), the *pro* gene lies in a separate reading frame, which is distinct from both *gag* and *pol* reading frames. Consequently, in these retroviruses there is also a second  $-1$  frameshift event to overcome the

second stop codon (located at the 3'-end of the *pro* gene), generating a full-length Gag-Pro-Pol precursor polyprotein (Coffin et al., 1997; Hatfield et al., 1992). The precursor polyproteins are all cleaved by the viral protease to the final-sized protein products, including the viral reverse transcriptase (RT).

MMTV was the first mammalian retrovirus identified and is the only infectious retrovirus with a distinct type B morphology, which was isolated as both endogenous and exogenous viruses (Moore et al., 1979; Ringold, 1983). Type B and type D retroviruses are the only retroviruses shown to accumulate in their virions the p30 protein, which possesses a dUTPase activity (Coffin et al., 1997; Hizi et al., 1997; Bergman et al., 1994; Koppe et al., 1994). This protein is unique in being a transframe protein, namely a protein containing sequences derived from two separate genomic translation reading frames. The amino-terminus of MMTV p30 has 94 residues translated from the *gag*-reading frame, which is also the precise sequence of the Gag nucleocapsid (NC) protein and the rest of the protein is encoded by the *pro* reading frame (Hizi et al., 1997). Thus, in these retroviruses there is a dual usage of gene regions for

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<sup>1</sup> Part of the results presented is in a partial fulfillment of a Ph.D. thesis at Tel Aviv University.

the translation of two independent and functionally different proteins (p30 and NC) with long overlapping polypeptide sequences. The viral protease is encoded by the rest of the *pro* gene that terminates at a stop codon (Hizi et al., 1997; Menendez-Arias et al., 1992).

The RT of MMTV is encoded by the *pol* gene open reading frame, which is distinct from both the *gag* and the *pro* reading frames (Moore et al., 1987). Our efforts to express in bacteria an enzymatically active recombinant MMTV RT have raised the possibility that this protein is also a transframe protein. A fully active recombinant RT could be obtained only after adding 27 non-*pol*-derived amino acid residues to the amino-terminus of the RT (Taube et al., 1998). These residues are encoded by the 3'-end of the *pro* gene and are present in the carboxyl-terminus of the MMTV protease (Moore et al., 1987; Hizi et al., 1997; Jacks et al., 1987). Based on the recombinant expression of MMTV RT, the length of the protease-derived sequences, necessary to restore the full enzymatic RT activity, ranges between 10 and 27 residues. Yet, this evidence is indirect. Moreover, one cannot rule out the possibility that in the specific case of MMTV RT, the protease-derived sequences are essential for the proper folding, solubility, and/or stability of only the bacterially expressed recombinant protein (and the mature RT in virions does not need to contain this extra sequence). Nevertheless, this argument is not supported by studies done with a variety of other recombinant retroviral RTs. So far, all recombinant RTs expressed in bacteria were fully active without any protease-derived sequences fused to their amino-termini. These include the RTs of HIV-1,<sup>2</sup> HIV-2, EIAV, MLV, FIV, PERV, and HTLV-1 (Hizi et al., 1991, 1998; Hizi and Hughes, 1988; Shaharabany et al., 1993; North et al., 1994; Owen et al., 1998; Avidan et al., 2003). In two other instances, of the expression of BLV RT and second method for the expression of HTLV-1 RT, the recombinant proteins were indeed transframe, since they include sequences from the *pro* gene. Nonetheless, these recombinant RTs do not include sequences that overlap the protease protein sequences, since in these retroviruses the *pro* reading frame ends at a stop codon, which is located at a significant distance downstream to the carboxyl-terminus of the proteases (Perach and Hizi, 1999; Trentin et al., 1998).

In the present study, we have investigated directly the origin of the amino-terminus of MMTV RT by analyzing the mature protein molecules in virions of MMTV. By using two anti-peptide-specific antisera, we have tested whether the amino-terminus of the viral RT contains protease-derived sequences. The data show that antibodies against the carboxyl-terminal segment of MMTV protease recognize

the same RT molecules as those detected by antibodies raised against a peptide from the *pol*-encoded molecule. This result supports the prediction that the virus-derived MMTV RT (similar to the recombinant RT) is covalently fused at its amino-terminus to the carboxyl-terminal peptide of the protease and is, hence, a transframe protein.

## Results and discussion

The rabbit antisera used in this study to identify the viral reverse transcriptase molecules were prepared against synthetic peptides with defined sequences derived from MMTV proteins. The antiprotease serum was prepared against the peptide with the sequence CDIKVRLMTDSPDDSQL, containing the last 17 residues of the carboxyl-terminus of MMTV protease (with an extra cysteine at the amino-terminus). The anti-RT serum was prepared against a 19-residue peptide with the sequence CFVIKKKSGK-WRLQLDLRA, which matches the sequence between amino acids 66 and 83 in the *pol* gene-encoded protein (with an extra cysteine at the amino-terminus). Both antisera were used in the two sets of experiments described below.

In the experiment described in Fig. 1, the proteins in the extract of the highly purified C3H strain MMTV virions were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western analysis performed with either the antiprotease or the anti-RT peptides sera. In parallel, the same electroblotted MMTV proteins were incubated with a control serum. As a positive antigen control, we have also used the purified recombinant MMTV RT (version 3 according to Taube et al., 1998), which was engineered to contain at its amino-terminus the last 27 amino acids from the carboxyl-terminus of the MMTV protease—in addition to a six histidines tag (to allow fast purification). This protein is 603 residues long plus the six histidines plus three other residues (derived from the expression vector), yielding a total length of 612 amino acids. As expected, the anti-RT serum recognizes the recombinant RT with an approximate molecular mass of 66 kDa. The same antiserum interacts with a viral protein, which is slightly smaller in size than the recombinant RT (Fig. 1A). This is probably the mature viral MMTV RT, which has an apparent molecular mass of about 65 kDa. In addition, a larger protein can be detected in the virus-derived proteins (lane 1), which probably represents the Gag-Pro-Pol precursor polyprotein, pr160 (Dickson and Peters, 1983). No reaction was apparent when the blotted viral proteins were incubated with a control rabbit serum (lanes 5 and 6). To further confirm the specificity of the antigen recognition by the anti-RT serum, the membrane was incubated with the antibodies in the presence of an excess of the purified recombinant MMTV RT. It is obvious that this immunoblotting process has substantially reduced the intensity of the reaction with the antibodies (that represents the amount of bound antibodies), suggesting that common antigenic epitopes are involved (lanes 3 and 4).

<sup>2</sup> Abbreviations used: HIV-1, human immunodeficiency virus type-1; HIV-2, human immunodeficiency virus type-2; MLV, murine leukemia virus; FIV, feline immunodeficiency virus; BLV, bovine leukemia virus; HTLV-1, human T cell leukemia virus-1; PERV, porcine endogenous retrovirus.

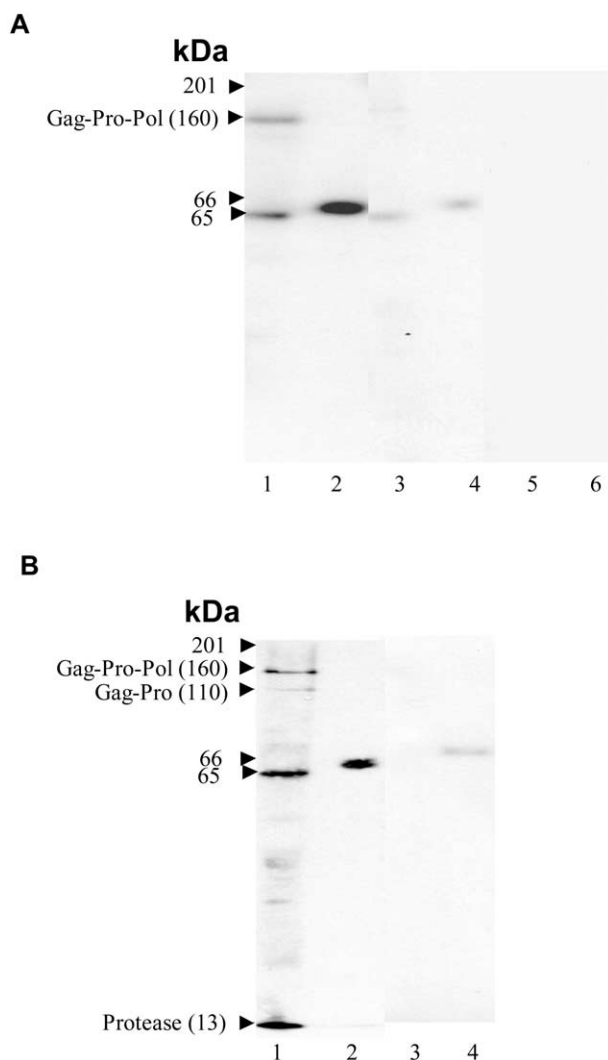


Fig. 1. Western blot analysis of viral and recombinant MMTV RT. Ten micrograms of purified virions of MMTV C3H (A, lanes 1, 3, and 5; and B, lanes 1 and 3) or 100 ng of the recombinant MMTV RT (A, lanes 2, 4, and 6, and B, lanes 2 and 4) was resolved by SDS–polyacrylamide gel electrophoresis and underwent Western analysis as described under Materials and methods. (A) The nitrocellulose filters were incubated with either the antiserum against the MMTV RT-derived peptide (lanes 1 and 2) or the same antiserum in the presence of 120 ng of purified recombinant MMTV RT (lanes 3 and 4) or with normal rabbit serum (lanes 5 and 6). (B) The membranes were incubated with either the antiserum raised against the protease-derived carboxyl-terminal peptide (lanes 1 and 2) or the same serum in the presence of 120 ng of the purified recombinant MMTV protease (lanes 3 and 4).

An experiment analogous to that shown in Fig. 1A was performed with the antiprotease serum (Fig. 1B). As expected, this serum recognizes the recombinant MMTV RT, confirming the presence of the protease-derived sequence in this protein. Interestingly, the same antiserum recognizes an approximately 65-kDa polypeptide in the virions (lane 1), which is very likely to be the same viral RT protein recognized by the anti-RT serum. This supports our hypothesis that sequences derived from the carboxyl-terminus of the protease are located within the viral RT molecules. The

antiprotease serum also interacts with an approximately 13-kDa polypeptide (which is probably the mature protease) and also recognizes two larger proteins. The larger one is presumably the Gag-Pro-Pol pr160 precursor polyprotein and the other one is likely to be the Gag-Pro pr110 precursor protein, which can both be found in virions of MMTV (Dickson and Peters, 1983). The specificity of the immunoreaction was confirmed by a competition experiment with an excess of purified recombinant MMTV protease. No detectable reaction is apparent, since the sequence of the recombinant protease has absorbed all antiprotease antibodies (lane 3). This clearly indicates that the antiprotease serum recognizes in this analysis only the protease-derived sequences. The conclusion from the experiment described in Fig. 1 is that there is evidence that the viral RT, similar to the recombinant protein, contains sequences that are derived from the carboxyl-terminus of the protease protein.

To further confirm the finding that both the anti-RT and the antiprotease sera recognize the same viral p65 RT protein, we have conducted the experiment described in Fig. 2. In this instance, the viral antigens were first immunoprecipitated by either the anti-RT or antiprotease antibodies. Then, the pellets were resolved by SDS–PAGE that was followed by a Western analysis, performed with the other antibody. Thus, the proteins immunoprecipitated with the anti-RT serum were treated in the Western analysis with the antiprotease serum and vice versa (immunoprecipitates with antiprotease serum were tested in the Western analysis with anti-RT antibodies). The results clearly show that the antiprotease antibodies recognized the viral p65 protein, immunoprecipitated by the anti-RT serum. Moreover, the anti-RT serum recognized the p65 protein, which had been precipitated by the antiprotease antibodies. These findings evidently indicate that both antisera interacted with the same p65 RT protein.

The data presented herein support our previous hypothesis that viral MMTV RT indeed contains sequences, derived from the carboxyl-terminus of the viral protease, at its amino terminus. Still, the results presented do not precisely define the amino-terminus of the viral RT. Our attempts to isolate the mature viral RT from polyacrylamide gels (on which the viral proteins have been resolved) and to identify its amino-terminus, either by microsequencing (using the Edman degradation) or by mass spectrometry (MS) of the RT-related peptides (generated by digestion with trypsin—see Materials and methods), did not lead to conclusive results. Extremely low amounts of these peptides were generated and their analysis was difficult and, for that reason, the sequences obtained were of low significance. This is probably due to the exceedingly low amount of the mature RT in MMTV virions. In the future, only a large-scale purification of this RT from large amounts of purified virions will probably allow the unambiguous determination of the precise amino-terminus of this protein. Consequently, this accurate amino-terminus can be speculated at present based only on the putative cleavage sequence specificities of

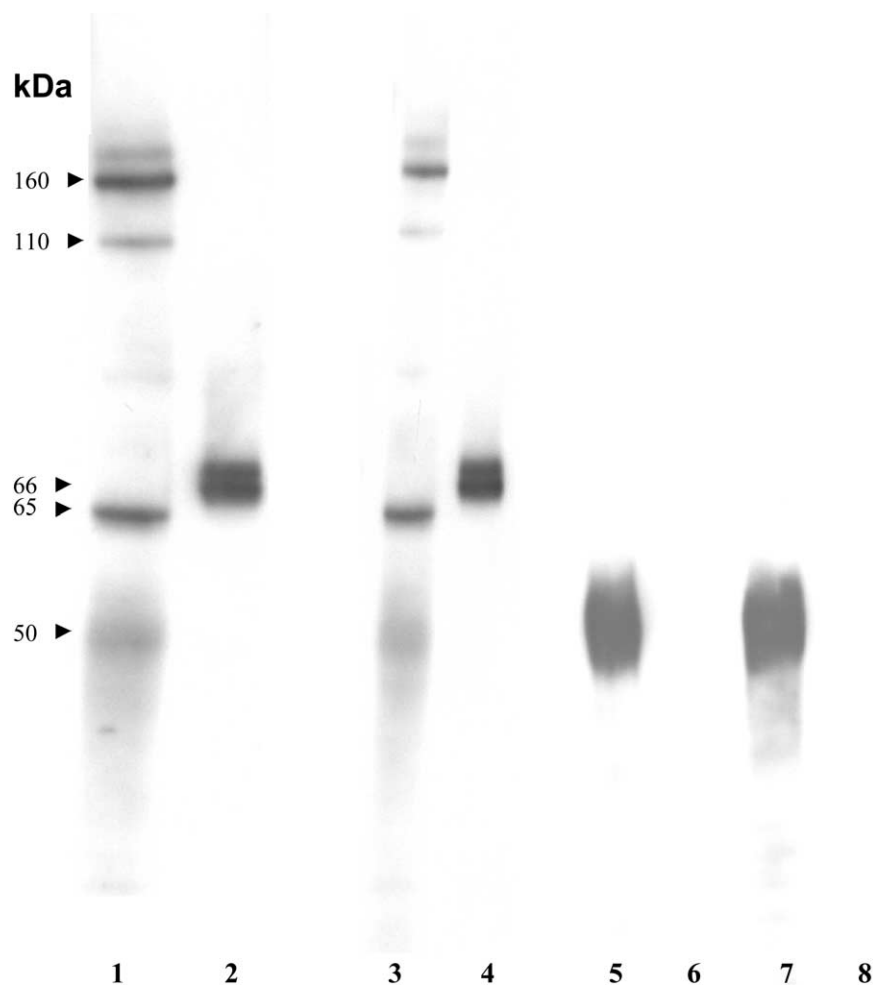


Fig. 2. Immunoprecipitation and Western blot analysis of viral and recombinant MMTV RT. Extracts prepared each from 250  $\mu$ g MMTV virions were immunoprecipitated by either the anti-RT peptide serum (lane 1), the antiprotease peptide serum (lane 3), or normal rabbit serum, followed by incubations with protein A–Sepharose. The washed immunoprecipitates were loaded onto SDS–polyacrylamide gels along with purified recombinant MMTV protease (lanes 2, 4, 6, 8) and resolved by electrophoresis. The proteins were electrotransferred from the gel onto nitrocellulose papers. The washed filters were incubated with either the antiprotease peptide serum (lanes 1, 2, 5, and 6) or the anti-RT peptide serum (lanes 3, 4, 7, and 8). The washed filters were incubated with goat anti-rabbit IgG antibodies and conjugated to horseradish peroxidase, as described under Materials and methods. The band of approximately 50 kDa represents the heavy chain of the rabbit IgG antibodies used in the immunoprecipitation step.

the MMTV protease. From these sequence specificities (Hizi et al., 1989, 1997; Menendez-Arias et al., 1992), we can still predict the precise amino-terminus of the viral RT by indicating the two most likely potential cleavages (Fig. 3). However, since the antiprotease serum was prepared against the last 17 residues of the protein, it seems to us that alternative cleavage site, marked as No. 1, is more likely (leaving all the 17 protease-derived residues within the

amino-terminus of the RT). According to this preference, 24 residues from the protease protein (starting with the sequence WGRDIMK) constitute the amino-terminus of the reverse transcriptase protein. Indeed, one of the tryptic peptides identified (unfortunately, only at a marginal significance) in the MS of the putative viral RT band might have started with this sequence (data not shown). Based on the assumption that both the recombinant and the viral MMTV



Fig. 3. The carboxyl-terminus of MMTV protease and the putative cleavage sites for the formation of amino-terminus of the RT. The last 30 residues of the protease (numbered backwards from the carboxyl-terminus) are shown along with the two alternative cleavage sites (marked by arrows 1 or 2). The site marked by an asterisk indicates the amino-terminus engineered previously for the recombinant MMTV RT (see in Taube et al., 1998).

RT versions share the same carboxyl-terminus, it is likely that the viral RT is 600 residues long (as opposed to the recombinant RT that is 612 residues long). The observed difference of approximately 1 kDa between the molecular masses of the recombinant and viral RTs (see Figs. 1 and 2) is in line with our suggestion. Moreover, this conclusion is also in agreement with our previous proposition that between 10 and 27 protease-derived carboxyl-terminal sequences should be added to the amino-terminus of the RT, to obtain a fully active recombinant RT (Taube et al., 1998).

The cleavage of the mature RT molecule from the precursor pr160 polypeptide is catalyzed, as the cleavage of all other Gag-Pro-Pol proteins, by the MMTV protease. According to the conclusion drawn from the experiments presented in this study, this cleavage should remove a significant portion of the carboxyl-terminus of the protease and, thus, fuse it to the amino-terminus of the RT. Such a cleavage will obviously lead to an inactivation by truncation of the protease molecules, which are derived from the Gag-Pro-Pol precursor polyprotein. Hence, it is conceivable that a sequence targeted by such a proteolytic cleavage should be recognized by the active protease only when it is a part of the Gag-Pro-Pol molecules and not as part of the Gag-Pro polyprotein precursor molecules.

The evidence presented in this article strongly supports the idea that in MMTV, a prototype of type B retroviruses, there is a dual usage of the protein sequences located at the carboxyl-terminus of the protease. This segment is an integral part of both the protease and the RT proteins. In MMTV, a similar overlapping sequence usage was also observed in the case of the NC and the p30 (dUTPase) proteins. Consequently, it is very likely that type B (and possibly type D) retroviruses have developed a unique dependence on such a mechanism (which is obviously very efficient in sequence usage, relative to the use of separate sequences). It will be of interest to study in the future the mechanisms and the factors that regulate this process in the context of the life cycle of the viruses.

## Materials and methods

### Materials

The C3H strain of MMTV grown on the Mm5mt cells was purified as described (Hizi et al., 1989). The recombinant RT of MMTV was expressed in bacteria and purified as described in detail previously (Taube et al., 1998). The MMTV protease was expressed by us in bacteria and purified (M. Entin-Meer and A. Hizi, unpublished results). The protease-expressing gene was introduced into a pT5M-based plasmid, which was engineered to express a six-histidines tag fused to the amino-terminus of the recombinant protein. The plasmid was used to transform the BL21(DE3)pLysS strain of *Escherichia coli*. Expression and purification of the protease with a six-histidines tag

were performed as described previously in detail for HIV RTs (Sevilya et al., 2001), except that the purification procedure involved the use of only a Ni-NTA affinity column. The two antipeptide rabbit antisera, described in the text, were generous gifts of Dr. S. Oroszlan from NCI. These two sera underwent heat inactivation for 30 min at 56°C.

### Western blot and immunoprecipitation

Ten micrograms of the purified MMTV virions or 100 ng purified recombinant MMTV RT were resolved by 9% SDS-PAGE, followed by electrotransferring of the proteins to nitrocellulose membranes. The blotted membranes were incubated overnight in 5% (w/v) milk powder in 30 mM Tris-HCl, pH 7.5, 125 mM NaCl, 0.1% Tween 20 (TBS-T) at 4°C (to block nonspecific binding). After blocking, the membranes were incubated for 1 h at room temperature with either the anti-RT peptide or the antiprotease peptide sera, diluted 1:600 in 5% milk powder in TBS-T, followed by intensive washings with TBS-T buffer. The membranes were then incubated for an additional hour with a horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody, purchased from Jackson Labs (diluted 1:40,000 in 5% milk in TBS-T), followed by further intensive washings with TBS-T. The washed membranes were then subjected for 1 min to the HRP substrate buffer (consisting of 2.5 mM luminol, 400 mM paracumaric acid, 100 mM Tris-HCl, pH 7.5, and 5.4 mM H<sub>2</sub>O<sub>2</sub>) and exposed to an X-ray film for periods of time ranging from 10 s to 1 min. The films were scanned on a scanner-video densitometer.

For the immunoprecipitation reactions, about 250 µg of purified MMTV virus was disrupted in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% (v/v) Triton X-100, and 150 mM NaCl. After extensive mixing, the virus extract was centrifuged and the supernatant was collected. Two to four microliters of the serum (either the anti-MMTV RT peptide or the anti-MMTV protease peptide) were then added, followed by 3 h incubation with rotation at 4°C. Four milligrams of protein A-Sepharose beads (Pharmacia) was then added to the reaction tubes and the whole mixtures were incubated with rotation at 4°C for an additional hour. After centrifugation, the pellets were washed three times with phosphate-buffered saline (PBS) and diluted in equal volumes of the 2× SDS-PAGE protein sample buffer. The samples were boiled for 2 min and the supernatants were collected, loaded on gels, and underwent PAGE and a Western analysis, as described above.

### In-gel proteolysis and mass spectrometry analysis

The Coomassie brilliant blue stained protein bands in the gels were cut with a clean razor blade and the proteins in the gel were reduced with 10 mM DTT and modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate. The gel pieces were treated with 50% acetonitrile in 10 mM

ammonium bicarbonate to remove the stain from the proteins followed by drying the gel pieces. These dried gel pieces were rehydrated with 10% acetonitrile in 10 mM ammonium bicarbonate containing about 0.1  $\mu\text{g}$  trypsin per sample. The gel pieces were incubated overnight at 37°C and the resulting peptides were recovered with 60% acetonitrile with 0.1% trifluoroacetate. These tryptic peptides were resolved by reverse-phase chromatography on 0.1  $\times$  300-mm fused silica capillaries (J&W, 100 micrometer ID) home-filled with porous R2 (Perspective). The peptides were eluted using an 80-min linear gradient of 5 to 95% acetonitrile with 0.1% acetic acid in water, at flow rate of about 1  $\mu\text{l}/\text{min}$ . The liquid from the column was electro-sprayed into an ion-trap mass spectrometer (LCQ, Finnigan, San Jose, CA). The MS was performed in the positive ion mode using repetitively full MS scan. This was followed by collision-induced dissociation (CID) of the most dominant ion selected from the first MS scan. The mass spectrometry data were compared to simulated proteolysis and CID of the proteins in the NR-NCBI database using the Sequest software (J. Eng and J. Yates, University of Washington, sold by Finnigan, San Jose, CA). The amino-terminal of the protein was sequenced on Peptide Sequencer 494A (Perkin-Elmer) according to manufacturer's instructions.

## Acknowledgments

We are deeply grateful to Dr. S. Oroszlan (from the NCI at Frederick, MD, USA) for the supply of the antisera used in this study and to Dr. Tamar Ziv (from the Smoler Protein Center at the Technion, Israel Institute of Technology, Haifa, Israel) for skillfully conducting the Edman degradation and the in-gel proteolysis and MS analyses. We also thank Dr. L. Menendez-Arias (from Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Spain) for valuable suggestions and Dr. S. Loya from our laboratory for critically reading the manuscript.

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