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Processing of In Vitro-Synthesized *gag* Precursor Proteins of Human Immunodeficiency Virus (HIV) Type 1 by HIV Proteinase Generated in *Escherichia coli*

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We expressed the *gag* and proteinase regions of human immunodeficiency virus (HIV) type 1 by transcription and translation in vitro. A synthetic RNA spanning the *gag* and *pro* domains gave primarily the unprocessed capsid precursor pr53. Efficient cleavage of this precursor was observed when the *gag* and *pro* domains were placed in the same translational reading frame, yielding equimolar amounts of the *gag* protein and of proteinase (PR). Expression of HIV type 1 PR in *Escherichia coli* as a fusion protein gave rapid autocatalytic processing to an HIV-specific protein of approximately 11 kilodaltons. HIV PR generated in *E. coli* specifically induced cleavage of the HIV capsid precursor, whereas deletion of the carboxy-terminal 17 amino acids of the proteinase rendered it inactive. Inhibitor studies showed that the enzyme was insensitive to inhibitors of serine and cysteine proteinases and metalloproteinases and was inhibited only by a very high concentration (1 mM) of pepstatin A.

The genomes of all replication-competent retroviruses, including human immunodeficiency virus (HIV), consist of three major genetic elements that are arranged in the order 5′-*gag-pol-env*-3′. The structural proteins of the nucleocapsid (encoded by the *gag* gene) and the viral replication enzymes (encoded by the *pol* gene) are exclusively synthesized as polyproteins that are proteolytically processed to the mature viral proteins by a virus-encoded proteinase (for reviews, see references 12 and 27). The products of the *pol* gene are generated only by translation of a large *gag-pol* polyprotein, and synthesis of this polyprotein is achieved by translational frameshifting in the 3′-terminal part of the *gag* gene (8). The viral proteinases (PRs [13]) from a number of avian (3, 26) and mammalian (28–30) retroviruses have been purified and characterized. These enzymes share limited sequence homology with the aspartic proteinases, especially in the region surrounding the proposed catalytic center (9, 19, 23), but are much smaller than the aspartic proteinases and contain only a single homologous aspartic acid residue. The proteolytic enzyme of HIV type 1 has been studied by expression of various parts of the *pol* reading frame in yeast cells (10), baculovirus (14), and *Escherichia coli* expression vectors (2, 4, 5, 16). The proteolytic activity maps to an 11-kilodalton (kDa) protein (99 amino acids) that is encoded immediately upstream of the viral reverse transcriptase and that appears to be generated by autocatalytic release from a larger precursor protein (2).

We report here the construction of an expression plasmid (pHIVproPII) that places the region from nucleotides (nt) 1640 to 2129 of the HIV cDNA under the control of the promoter and untranslated region of bacteriophage T7 gene 10. This vector was constructed by digestion of plasmid pBHλ10 (20) with *Eco*RI and *Kpn*I, followed by digestion with *Dra*I. The 788-base-pair fragment (nt 1334 to 2122 of HIV cDNA) was isolated and ligated to complementary oligonucleotides that restored the PR cDNA sequence and provided two stop codons followed by an *Eco*RI site. The resulting fragment was then recut with *Bgl*II (nt 1640 of HIV cDNA) and *Eco*RI, and the 497-base-pair fragment was isolated and ligated with the *Bam*HI-*Eco*RI fragment of pAR2106 (21). The expected primary translation product from this vector is a fusion protein containing 12 amino acids derived from phage T7 gene 10, followed by 163 amino acids from the *pol* open reading frame of HIV type 1, starting at codon 5 of the *pol* reading frame and ending immediately upstream of the amino terminus of the reverse transcriptase. The expression plasmid was introduced into *E. coli* BL21(DE3), which contains an integrated copy of the T7 RNA polymerase gene under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *lac* UV5 promoter (22). In parallel experiments we transformed *E. coli* BL21(DE3) with the vector plasmid pAR2106 (containing no HIV sequences) and with plasmid pHIVproP, which is similar to pHIVproPII but has a deletion of the 17 3′-terminal codons of the proteinase gene. This plasmid was constructed by ligating the *Bgl*II-*Hin*cII fragment (nt 1640 to 2078 of HIV cDNA) with the *Bam*HI-*Eco*RI (filled) fragment of pAR2106.

The products from these expression plasmids after induction and labeling with [^{35}S]methionine are shown in Fig. 1A. Bacteria carrying the vector plasmid (pAR2106) primarily produced β-lactamase, which is encoded on the plasmid in the same orientation as the T7 promoter. Induction of bacteria harboring pHIVproP and pHIVproPII gave additional products of approximately 17 and 11 kDa, respectively. Immunoblot analysis of the bacterial lysates with a polyclonal antiserum against HIV PR (1) specifically detected the 17-kDa protein in the pHIVproP lane and the 11-kDa protein in the pHIVproPII lane (Fig. 1B). No trace of a 19-kDa precursor protein corresponding to the 175-amino-acid primary translation product from pHIVproPII was seen. The size of the immunoreactive protein agrees well with the reported size for mature HIV PR (99 amino acids [2]). In a separate experiment we labeled bacteria carrying pHIVproPII with [^{3}H]isoleucine and subjected the gel-purified 11-kDa protein to automated Edman degradation. The results obtained (data not shown) agreed with the reported amino

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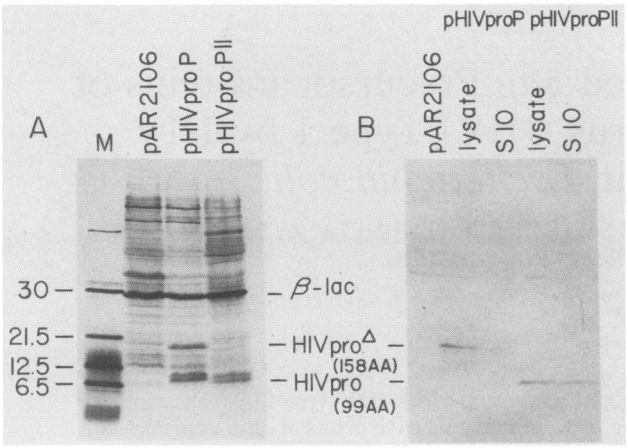

FIG. 1. Expression of the HIV proteinase in *E. coli*. Bacteria carrying expression vectors were grown in unsupplemented M9 medium (15) to an A_{600} of 1.0, and duplicate cultures were induced with 0.4 mM IPTG for an additional 90 min. [^{35}S]methionine was added to a final concentration of 10 μCi/ml, and cells were labeled for 30 min. The bacteria were collected by centrifugation in an Eppendorf centrifuge for 2 min, washed in 20 mM Tris hydrochloride (pH 7.5)–0.1 M NaCl (TN), recentrifuged, and suspended in TN. The suspensions were adjusted to 1% sodium dodecyl sulfate (SDS) and 2.5 mM DTT and boiled for 3 min. Lysates were centrifuged for 2 min, and supernatants were analyzed on a 10 to 20% gradient polyacrylamide-SDS gel. Subsequently, proteins were transferred to nitrocellulose for 3 h at 0.2 A as described previously (11). After transfer the nitrocellulose was cut in two parts. (A) The nitrocellulose paper was dried and exposed to Kodak XAR-5 film for 18 h. Lane M represents a mixture of ^{14}C-methylated proteins (low-molecular-weight kit) obtained from Amersham Corp. The positions of β-lactamase (β-lac), the truncated fusion protein, and the mature HIV PR are indicated. (B) The nitrocellulose was subjected to immunoblot analysis by using polyclonal antiserum against the HIV PR (1), essentially as described previously (11). Detection was with goat anti-immunoglobulin G (rabbit) coupled to alkaline phosphatase (Tago Immunochemicals) and with indolyl phosphate/Nitro Blue Tetrazolium chloride as the indicator-substrate system. Lysate, Total bacterial lysates; S10, supernatant after a 10,000 × *g* centrifugation; AA, amino acids.

terminus of HIV PR (Pro-69 of the *pol* reading frame [2]). We conclude that expression of the 175-amino-acid fusion protein in *E. coli* led to rapid and complete release of the mature 99-amino-acid HIV PR, presumably by intramolecular cleavage. Deletion of the 17 carboxy-terminal amino acids of the proteinase abolished proteolytic processing completely, and only the primary translation product was detected in an immunoblot analysis (158 amino acids; Fig. 1B, lane pHIVproP, lysate). The PR precursor truncated by 17 residues ends at a Val residue two amino acids upstream of a sequence of two hydrophobic amino acids (Ile-Ile in HIV) followed by a Gly residue that is completely conserved, not only in all retroviral proteinases but also in the aspartic proteinases (9, 18). Pearl and Taylor (18) suggested a structural model for HIV PR based on similarities with known cellular aspartic proteinases. In this model the Ile-Ile-Gly sequence is part of an antiparallel β strand that lies next to the proposed catalytic center (LLDTG in HIV), with the Gly residue interacting with the presumed active-site Asp. It is therefore quite possible that restoration of this β strand, still leaving a carboxy-terminal deletion of 11 amino acids, will restore proteolytic activity, and future experiments will address this question.

In order to biochemically characterize and purify HIV PR expressed in *E. coli* we developed a *trans* assay for the enzyme. This assay makes use of an in vitro transcription-translation system to synthesize substrates for HIV PR, a strategy successfully developed for the characterization and purification of the poliovirus proteinases (11, 17; M. J. H. Nicklin, K. Harris, P. V. Pallai, and E. Wimmer, J. Virol., in press). To generate the transcription vectors, we cloned a segment of the HIV cDNA, preceded by a promoter of phage T7, into a pBR322 derivative. This plasmid (pHIVg/p; Fig. 2A) was constructed by ligating the *Eco*RI-*Eco*RI fragment of pBHλ10 (nt 221 to 4228 of the HIV cDNA) with the small *Eco*RI-*Pst*I fragment of pBR322 (nt 4361 to 3609) and with the *Pst*I-*Eco*RI fragment of pMN39 (containing the promoter of phage T7 gene 10 followed by an *Eco*RI site inserted counterclockwise into the *Bam*HI site of pBR322; M. J. H. Nicklin and E. Wimmer, unpublished data). This vector was then modified to introduce two stop codons immediately downstream of the proteinase coding region (pHIVg/pII; Fig. 2A). Runoff transcription of the linearized plasmids in vitro with T7 RNA polymerase (25) yielded synthetic mRNAs that contained a segment of the HIV 5′-untranslated region from nt 221 to 333, the coding region for the *gag* precursor, and all or part of the coding region for PR. Synthetic RNAs were translated in rabbit reticulocyte lysates (RRLs; Promega Biotec) in the presence of [^{35}S] methionine as described by the supplier. Translation of a synthetic mRNA derived from pHIVg/pII, containing the complete *gag* and PR region, gave mainly the *gag* precursor pr53 (Fig. 2B, lane 1), which could be immunoprecipitated with a polyclonal anti-*gag* serum (data not shown) and with a monoclonal antiserum against the major structural protein p24 (data not shown). The shorter translation products probably correspond to either internal initiation products or prematurely terminated polypeptide chains. The full-length translation product (62 kDa), which is only observed when frameshifting into the *pol* reading frame occurs, was barely visible (Fig. 2B, lane 1). In addition, there was a weak protein band migrating where authentic p24 of HIV would be expected. This product was absent in a translation mixture programmed with synthetic mRNA transcribed from pHIVg/p linearized with *Hin*cII (Fig. 2B, lane 7), which is identical to g/pII RNA except for the deletion of the 17 3′-terminal codons of PR. Cleavage of the capsid precursor to p24 upon the translation of the g/pII transcript was, however, very weak, and further incubation of the translation mixtures for an additional 60 min (Fig. 2B, lane 2) or 5 h (lane 3) gave no further processing. Similarly, dilution of the mixture 1:10 and incubation for an additional 5 h (Fig. 2B, lane 4) yielded no increase in cleavage products.

Lack of efficient autocatalytic cleavage of this *gag*-PR precursor may have been due to an insufficient concentration of PR in the reaction mixture. We therefore constructed a transcription-translation vector that produces equimolar amounts of *gag*-related precursor and PR independent of translational frameshifting. The plasmid, pHIVFSII, was generated from pHIVg/pII by introducing four additional nucleotides (GATC) at a unique restriction site just four codons downstream of the normal frameshift site (Fig. 2A). This mutagenesis changed the nucleotide sequence from AAG ATC TGG to AAG ATC *GAT C* TG G and therefore leads to an insertion of an Asp codon (GAT) and to a switch from the *gag* reading frame (TGG = Trp) to the *pol* reading frame (CTG = Leu). This switch occurs only four codons after the start of the *pol* reading frame, and the primary translation product therefore closely resembles the product generated in vivo by translational frameshifting. The trans-

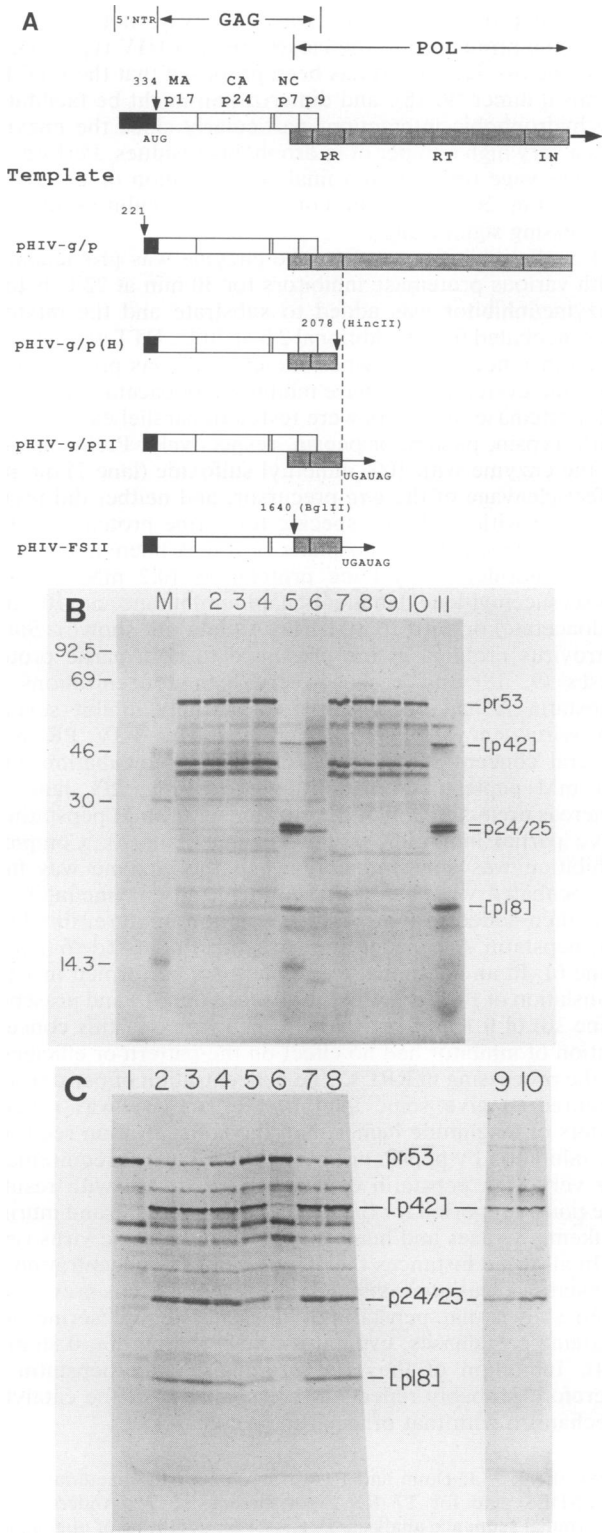

FIG. 2. (A) Map of the sequences contained in the synthetic mRNAs used for translation in vitro. The template is indicated on the left. At the top, a segment of the HIV genome is shown, consisting of the 5′ untranslated region (■), the *gag* region (□), and part of the *pol* region (▨). Proteins derived from different reading frames are in different lines. The cleavage products from the *gag* precursor and from the *pol* segment of the polyprotein (designated in the old and new nomenclature; 7, 13) are also indicated. Cleavage sites are depicted as vertical lines. In the representation of the transcripts, relevant restriction endonuclease cleavage sites used for the linearization of the template (*Hin*cII) or for the generation of a frameshift mutation (*Bgl*II) are indicated. A stop codon at the 3′ end of the HIV-specific sequences is indicated by the nucleotide sequence. (B) Products of translation of synthetic RNAs in RRLs. Samples of translation mixtures (1 to 3 μl) were either mixed directly with sample buffer or incubated with or without different bacterial extracts and then mixed with sample buffer and analyzed on a 12.5% polyacrylamide-sodium dodecyl sulfate (SDS) gel. The dried gels were exposed to Kodak XAR-5 film for 16 h at −80°C. The left lane corresponds to a translation with no added mRNA, and lane M corresponds to a mixture of ^{14}C-methylated proteins, obtained from Amersham (note that the methylated ovalbumin [molecular weight, 46,000] in the marker lane was partially degraded and migrated aberrantly). For cleavage assays, pellets of bacterial cells corresponding to 100 ml of culture which had been induced with 0.4 mM IPTG for 2 h and stored at −80°C were suspended in 5 ml of TN (20 mM Tris hydrochloride [pH 7.5], 0.1 M NaCl) containing 5 mM EDTA and 5 mM DTT. The cells were lysed by sonication and kept on ice. Cleavage reactions were carried out in a final volume of 10 μl, using 1 to 3 μl of translation mixture as substrate and 1 μl of bacterial lysate. The final buffer was 20 mM Tris hydrochloride (pH 7.5)–0.1 M NaCl–5 mM EDTA–5 mM DTT. Reaction mixtures were incubated for 60 min at 30°C. Lanes: 1, g/p(H) RNA, no incubation; 2 g/p(H) RNA, 1 h at 30°C; 3, g/p(H) RNA, 5 h at 30°C; 4, g/p(H) RNA, 1:10 dilution in 20 mM Tris hydrochloride (pH 7.5)–0.1 M NaCl–5 mM EDTA–5 mM DTT, incubation for 5 h at 30°C; 5, g/p(H) RNA, incubation with bacterial extract carrying pHIVproPII; 6, FSII RNA, no incubation; 7, g/pII RNA, no incubation; 8, g/pII RNA, 1 h at 30°C; 9, g/pII RNA, incubation with bacterial extract carrying pAR2106; 10, g/pII RNA, incubation with bacterial extract carrying pHIVproP; 11, g/pII RNA, incubation with bacterial extract carrying pHIVproPII. (C) g/p(H) RNA was translated in RRLs, and the products were either analyzed directly on a 12.5% polyacrylamide-SDS gel (lane 1) or incubated with extracts from bacteria carrying pHIV proPII (lanes 2 to 8). Bacteria were lysed into TN and centrifuged at 10,000 × *g* for 15 min. The supernatant was diluted 1:10 in TN and was made 2.5 mM DTT, except that in lane 3. Portions were then preincubated with or without pepstatin A for 30 min at 22°C, followed by 2-h cleavage reactions at 30°C. Lanes: 1, no addition of enzyme; 2, addition of bacterial lysates carrying pHIVproPII (1:10 dilution) with DTT present; 3, as in lane 2 but no addition of DTT; 4, preincubation with 0.1 mM pepstatin A; 5, preincubation with 1 mM pepstatin A; 6, preincubation with 1 mM pepstatin A and addition of pepstatin A to the cleavage reaction mixture to give a final concentration of 1 mM; 7, preincubation with 10% dimethyl sulfoxide; 8, cleavage reaction performed in the presence of 1% Triton X-100; 9, translation of FSII RNA; 10, translation of FSII RNA in the presence of 0.1 mM pepstatin A.

lation products from a reticulocyte mixture programmed with FSII RNA are shown in Fig. 2B, lane 6. This translation mainly gave proteins of 42, 24, 18, and 13 kDa but only a weak band corresponding to the full-length translation product. Thus, the majority of the *gag*-PR precursor was rapidly and efficiently cleaved in this system. All translation products were specifically immunoprecipitated with anti-*gag* serum, and the 42- and 24-kDa proteins reacted specifically with anti-p24 serum (data not shown). The 24-kDa protein corresponds to the mature structural protein p24, and the 42-kDa product is likely to contain the sequences of the amino-terminal *gag* protein p17 in addition to the p24 sequences.

These experiments relate to the question of why proteolytic processing takes place only at a specific time in the replicative cycle and whether the viral proteinase is inactive immediately after polyprotein synthesis and requires activation by a cofactor of viral or cellular origin. Whatever factors are required for processing are clearly present in our in vitro

system, since rapid and efficient cleavage occurs. It is possible that cleavage depends on a certain minimal concentration of enzyme, especially if the enzyme needs to form a dimer, as has been proposed (18), and that this "minimal cleavage concentration" is only achieved in vivo once the enzyme reaches a certain (virus-induced?) compartment. This hypothesis could be supported by our observation that equimolar expression of substrate and enzyme gives efficient cleavage.

In further experiments we used the translation product from g/p(H) RNA as a substrate for HIV PR generated in *E. coli*. RRLs, programmed with g/p(H) RNA, were either analyzed directly after translation (Fig. 2B, lane 7) or incubated for 60 min under the following conditions: (i) in buffer without addition of bacterial extracts (lane 8), (ii) in the presence of bacterial lysates carrying the vector plasmid pAR2106 (lane 9), or (iii) in the presence of lysates carrying the HIV expression plasmid pHIVproP (yielding an HIV PR with the C-terminal deletion; lane 10) or pHIVproPII (yielding mature HIV PR; lane 11). Cleavage of the pr53 *gag* precursor was observed only when the translation products were incubated with bacterial extracts containing mature HIV PR (lane 11). The capsid precursor was completely stable on incubation in buffer alone, with *E. coli* extracts not containing HIV-specific sequences, or with a truncated HIV PR whose 17 carboxy-terminal amino acids had been deleted. The cleavage observed in lane 11 was complete, and all but one cleavage product comigrated with the products synthesized in vitro from FSII RNA (Fig. 2B, lane 6). The only difference was a protein migrating slightly more slowly than the p24 capsid protein (designated p24 in Fig. 2B). This protein was also observed in a previous study (10) in which HIV *gag* and PR regions were expressed in yeast cells. The 42- and 24-/25-kDa products reacted with a monoclonal antibody against p24 (data not shown), and all cleavage products were immunoprecipitated by a polyclonal antiserum prepared against HIV *gag* proteins (data not shown). It has been reported that p24 is generated from a slightly larger intermediate precursor by removal of 14 C-terminal amino acids (7). The 25-kDa product observed in our experiment probably corresponds to this intermediate.

The cleavage of in vitro-synthesized *gag* precursor protein can be used as a *trans* assay for the biochemical characterization of HIV PR expressed in *E. coli*. For this purpose, a translation mixture programmed with g/p(H) RNA served as a substrate (Fig. 2C, lane 1) while an S10 supernatant from bacterial lysates after expression of the pHIVproPII vector was used to provide the enzyme (Fig. 2C, lanes 2 to 8). In a preliminary experiment we determined that most of the HIV-specific proteolytic activity was contained in an S10 supernatant (data not shown). Bacteria carrying pHIVproPII were lysed into 20 mM Tris hydrochloride–0.1 M NaCl (pH 7.5) without a reducing agent, an S10 fraction was prepared, and dithiothreitol (DTT) was added to 2.5 mM except when indicated. The S10 fraction (enzyme) was then diluted 1:10 into lysis buffer (with or without DTT). This was done because the enzyme concentration used in the previous experiment gave complete cleavage and minor inhibitory effects might therefore be missed. Accordingly, incubation of the RRL translation products (substrate; Fig. 2C, lane 1) with a bacterial S10 fraction (diluted 1:10) gave incomplete cleavage of the precursor protein, and the expected products were observed (Fig. 2C, lane 2). The efficiency and pattern of cleavage were identical regardless of whether DTT was present (Fig. 2C, lane 2) or absent (lane 3), an observation suggesting that the enzyme does not belong to the family of cysteine proteinases. This was, however, not surprising since the proteinase of the closely related HIV type 2 lacks cysteine residues (6). It has been proposed that the HIV PR forms a dimer (9, 18), and dimerization might be facilitated by hydrophobic interaction, particularly since the enzyme has a very high number of hydrophobic residues. Performing the cleavage reaction in a final concentration of 1% Triton X-100 (Fig. 2C, lane 8) did not, however, inhibit proteolytic processing significantly.

For the inhibition studies, the enzyme was preincubated with various proteinase inhibitors for 30 min at 22°C before enzyme/inhibitor was added to substrate and the mixture was incubated for an additional 2 h at 30°C. DTT was present in all instances except when the enzyme was preincubated with the cysteine proteinase inhibitors iodoacetate and E64. All proteinase inhibitors were tested in parallel experiments with trypsin, papain, or pepsin, respectively. Preincubation of the enzyme with 10% dimethyl sulfoxide (lane 7) did not affect cleavage of the *gag* precursor, and neither did preincubation with inhibitors specific for serine proteinases (0.5 mM diisopropylfluorophosphate or 3 mM phenylmethylsulfonyl fluoride) or cysteine proteinases (0.2 mM L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane or 10 mM iodoacetate) or with 10 mM EDTA (data not shown). Since retrovirus proteinases are presumed to be aspartic proteinases (9, 18) and because very high concentrations of pepstatin A had been shown to partially inhibit several retrovirus enzymes (9), we preincubated HIV PR with several concentrations of pepstatin A. Preincubation with 0.1 mM pepstatin A had little effect (Fig. 2C, lane 4), whereas preincubation of the enzyme with 1 mM pepstatin A gave partial inhibition of the enzyme (lane 5). Complete inhibition was achieved only when the enzyme was first preincubated with 1 mM pepstatin A and enzyme/inhibitor was then added to the cleavage reaction mixture, in which the pepstatin A concentration was also adjusted to 1 mM (lane 6). In an additional experiment we performed in vitro translation of FSII RNA in the absence (lane 9) and presence (lane 10) of 0.1 mM pepstatin A, showing that this concentration of inhibitor had no effect on the pattern or efficiency of the processing in RRLs. The concentration of pepstatin A required to give some inhibition of activity was several orders of magnitude higher than the concentration required for inhibition by pepsin or even renin (24). This requirement for very high pepstatin concentrations agrees with results previously reported for the proteinases of bovine and murine leukemia viruses and human T-cell lymphotropic virus type I. In all these instances the 50% inhibitory concentration of pepstatin A was approximately 0.5 mM (9). It has previously been shown that pepstatin A does not inhibit serine and cysteine proteinases, even at a concentration of >0.36 mM (24). Inhibition of the HIV proteinase with pepstatin A therefore probably reflects a true similarity of the catalytic mechanism with that of aspartic proteinases.

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