Mapping of Nucleic Acid Binding in Proteolytic Domains of HIV-1 Reverse Transcriptase

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ABSTRACT: Human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) and its domain fragments were used to map nucleic acid binding sites within the enzyme. Discrete domain fragments were produced after the digestion of three forms of RT (p66, p66/p51 heterodimer, and p51) with V8 protease or trypsin, and the primary structure of each domain fragment was mapped by both immunoblotting and N-terminal amino acid sequence analysis. These domain fragments represent N-terminal, middle, or C-terminal regions of RT. Using Northwestern or Southwestern blotting assays, the domain fragments were evaluated for nucleic acid binding. In this technique, RT proteins are electroblotted onto the membrane and renatured after SDS–PAGE; the proteins are then probed with the primer analogues ^{32}P-labeled d(T)$_{16}$ or ^{32}P-labeled tRNA^{Lys,3}. A V8 protease domain fragment spanning residues 195 to ~300 (p12), which was found earlier to be UV cross-linked to the primer in intact RT [Sobol *et al.* (1991) *Biochemistry 30*, 10623–10631], showed binding to both nucleic acid probes. We first localized nucleic acid binding in p66 to an N-terminal domain fragment of residues 1 ≅ 300. By contrast, a C-terminal domain fragment termed p30$_{(303\cong560)}$ did not show nucleic acid binding. To investigate the role of the region just N-terminal to residue 303, an expression vector named pRC-35 encoding residues 273–560 was constructed. We purified the corresponding expressed protein, p35, and found that this protein binds to tRNA^{Lys,3}, demonstrating that residues 273–302 are able to confer nucleic acid binding to the binding-negative C-terminal segment spanning residues 303 ≅ 560. Further, an additional domain fragment corresponding to residues 1 ≅ 230 (p29) was found to have nucleic acid-binding capacity. These results indicate that RT nucleic acid binding occurs in at least two domains in the N-terminal half of p66. The results appear in good agreement with the model of template–primer bound to the p66/p51 heterodimer, proposed by Kohlstaedt *et al.* [(1992) *Science 256*, 1780–1789].

Human immunodeficiency virus type-1 (HIV-1),[1] like other retroviruses, replicates by reverse transcription of the plus strand RNA genome into the double-stranded proviral DNA. This process requires the RNA-dependent DNA polymerase, known as reverse transcriptase [(Temin & Mizutani, 1970; Baltimore, 1970; reviewed in Vaishnav and Wong-Staal (1991)]. RT purified from HIV-1 virions is a dimer of a ~66 000 = M_r polypeptide (p66) and a ~51 000 = M_r polypeptide (p51), which is a C-terminal truncated form of p66 [Chandra *et al.*, 1986; Hansen *et al.*, 1988; see Jacobo-Molina and Arnold (1991) for a recent review]. The viral encoded protease specifically cleaves p66 to yield p51 (Lightfoote *et al.*, 1986; Starnes & Chang, 1989). Recently, we separately overexpressed p66 and p51 from HXB2 proviral DNA in *Escherichia coli* and purified these proteins in large amounts (Becerra *et al.*, 1991). We then made use of these proteins along with truncation mutants to demonstrate that the central and C-terminal regions of p66 are involved in dimer formation.

Reverse transcription of the viral genome is initiated *in vivo* from the 3′ hydroxyl end of host cell tRNA^{Lys,3} hybridized near the 5′ end of the viral RNA genome at the primer-binding site (Taylor & Illmensee, 1975; Harada *et al.*, 1975). The sequence of the HIV-1 primer-binding site (Ratner *et al.*, 1985) is complementary to the last 18 nucleotides at the 3′ terminus of tRNA^{Lys,3} (Weiss *et al.*, 1985). Previous work by Darlix and co-workers and others [for review, see Wilson and Abbotts (1992)] demonstrated that recognition of tRNA^{Lys,3} by HIV-1 RT occurs, in part, through interactions of the anticodon loop with the enzyme.

Kinetic studies of recombinant HIV-1 RT indicated that the reaction pathway for DNA synthesis is ordered; i.e., the enzyme binds to the template–primer to form the first complex in the reaction scheme (Majumdar *et al.*, 1988; Huber *et al.*, 1989). However, in the presence of substrate analogues for the primer, the free enzyme can bind to primer analogues in the absence of the template (Majumdar *et al.*, 1989), albeit with much lower K_A than binding to template–primer (Beard & Wilson, 1993). We and others have shown recently that RT complexed with primer analogues, oligo d(T)$_8$ or oligo d(T)$_{16}$, can be covalently cross-linked using UV light (Sobol *et al.*, 1991). We used this approach to elucidate a region in the primary structure of HIV-1 RT that is in close proximity

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[1] Abbreviations: HIV-1, human immunodeficiency virus type-1; RT, reverse transcriptase; pol, DNA polymerase; DTT, dithiothreitol; d(T)$_8$, d(T)$_{16}$, oligomers of deoxythymidylate; dNTP, deoxynucleoside triphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; AZT, 3′-azidodeoxythymidine; ddI, dideoxyinosine.

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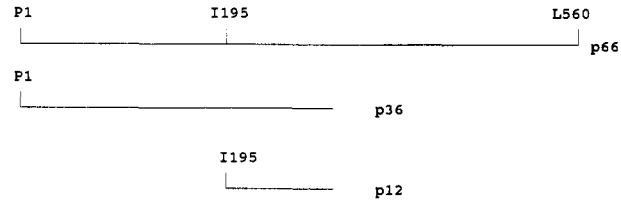
to bound oligo d(T). Two V8 protease peptides, p36 and p12, contained the cross-linked primer as follows:



Modak and collaborators also labeled a primer-binding region of HIV-1 RT using $d(T)_{15}$ and UV-light as cross-linker (Basu *et al.*, 1992). A single cross-linked tryptic peptide (residues 288–307) was identified, and L^{289}-T^{290} and L^{295}-T^{296} appeared to be sites of cross-linking.

A crystal structure at 3.5-Å resolution of HIV-1 RT was published recently by Kohlstaedt *et al.* (1992). Their structural model indicates that L^{289}-T^{290} and L^{295}-T^{296} are in the so-called "thumb" region and are part of a postulated DNA–RNA template–primer binding groove of the enzyme. Kohlstaedt *et al.* proposed that the p66 subunit in the heterodimer conformation of RT catalyzes the polymerase reaction, while the p51 subunit forms part of the binding site for the tRNA primer, as well as a portion of the template–primer binding site.

We describe here the detailed domain mapping of HIV-1 RT by proteolytic cleavage. We have localized nucleic acid-binding sites to two regions in the N-terminal half of the primary structure of the protein. Using Northwestern or Southwestern assays, we have shown that C-terminal domain fragments, residues 303–560 and 427–560, do not bind our nucleic acid probes, while N-terminal domain fragments $p36_{(1\cong300)}$, $p29_{(1\cong230)}$, or $p12_{(195\cong300)}$ bind to the nucleic acid probes. The N-terminal domain fragment $p29_{(1\cong230)}$ produced by trypsin proteolysis of p51 shows binding to nucleic acid, and an overexpressed peptide, p35, containing residues 273–560 also binds nucleic acids. These results indicate that nucleic acid binding is not limited to one region of RT and permits localization of two binding regions in the N-terminal half of p66. The localization of nucleic acid-binding sites in RT is an important step toward the design of inhibitors that block this key step in RT function.

MATERIALS AND METHODS

Materials. α-[^{32}P]UTP (3000 Ci/mmol) and Rainbow protein molecular weight markers were purchased from Amersham Corp. Poly(A), $d(T)_{16}$, and NICK columns were purchased from Pharmacia Biochemicals Inc.; *Staphylococcus aureus* V8 protease and trypsin were from Worthington Biochemical Corp.; bovine serum albumin, polyvinylpyrrolidone and Ficoll were from Sigma Chemical Co., and monoclonal antibody mAb NEN was purchased from New England Nuclear, Inc. Monoclonal antibodies mAb 19, mAb 42, mAb 21, and mAb 50 (Ferris *et al.*, 1990) were a gift from Dr. Stephen H. Hughes (BRI Basic Research Program, NCI—Frederick Cancer Research and Development Center, Frederick, MD).

Preparation of Proteins. p66 (residues Pro^1-Leu^{560}), p66/p51 complex, and p51 (residues Pro^1-Leu^{425}-Cys; Cys^{426} is different from the authentic RT sequences) were purified as described (Becerra *et al.*, 1991). p15 (Meth-Asn-Glu-Leu followed by amino acids Tyr^{427}-Leu^{560}) purification was carried out according to Becerra *et al.* (1990).

Construction of Plasmid for Production of p35. Construction of expression vector pRC-35 is described in Becerra *et al.* (1993). Construct pRC-35 was derived from pRCΔ-RT*2, an expression vector for full-length reverse transcriptase with precise RT coding sequence and a *Sma*I site at N813 (nucleotide numbering starts at the first base of the first codon for RT, N1). The *Sma*I site was generated via oligonucleotide-directed mutagenesis of the corresponding M13-RT (Becerra *et al.*, 1991), an M13mp18-derived subclone containing the precise coding sequence of RT. Restriction digestion of pRCΔ-RT*2 with *Msc*I (N70) and *Sma*I (N816) followed by ligation of its blunt ends produced transformants in *E. coli* [pRK-248cIts] containing plasmid pRC-35. This plasmid has RT-truncated sequences under the control of the λp_L promoter. The deletion in pRC-35 encompasses bases N73–N816 and maintained the RT coding sequence in-frame. Amino acid residues specified in pRC-35 are as follows: RT 1-24 and 273–560. Expression of the RT sequences from vector pRC-35 was performed as before (Becerra *et al.*, 1991).

Purification of p35. A total of 10 g of *E. coli* cells containing pRC-35, induced 3 h at 42 °C in a fermentor, was suspended in 100 mL of lysis buffer (25 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20% glycerol, 1% Triton x-100, 1 mM EDTA, and 2 mM DTT). All the purification buffers contain protease inhibitors (1 mM PMSF and 1 μg/mL pepstatin A). The cell suspension was probe-sonicated with four bursts, 30-s pulse, and 1-min pause with the tube immersed in an ice-water bath using a sonicator (Model W-225, Ultrasonics, Inc.). The sonicated cell suspension was centrifuged at 10000*g* for 10 min. The supernatant fraction was dialyzed against buffer A (50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 75 mM NaCl at 4 °C for 3 h. The dialyzed extract was centrifuged at 10000*g* for 10 min, and the supernatant was layered on a Q-Sepharose fast-flow (Pharmacia) column (30-mL bed column) equilibrated with buffer A containing 75 mM NaCl. The flow-through fraction was collected which contained all the p35 protein as judged by Western blotting using mAb 50.

The flow-through fraction was passed through a 0.22-μm filter (MILLEX-GV, Millipore Corp.). The filtered solution was layered on a mono-S (Pharmacia) column (HR 5/5) equilibrated with buffer A containing 75 mM NaCl. The column was washed with equilibration buffer and elution of p35 was accomplished with a gradient of 75–500 mM NaCl in buffer A at 1.0 mL/min. One-milliliter fractions were collected, and peak fractions containing p35 were pooled.

Preparation of $tRNA^{Lys,3}$ Gene Construct. Two oligonucleotides were synthesized by Operon Technologies, Inc. (Alameda, CA), a 109-base deoxynucleotide and a 20-base deoxynucleotide which served as template and primer, respectively, for generating a double-stranded molecule with Klenow fragment. Nucleotide sequence of the template strand, 5′-GAATTCATGC AT*GGCGCCCG AACAGGGACT TG-AACCCTGG ACCCTCAGAT TAAAAGTCTG ATGCTC-TACC GACTGAGCTA TCCGGGC*TAT AGTGAGTCGT ATTACTGCA-3′ contained the following sites from the 5′ end: an *Eco*RI restriction site, a *Nsi*I restriction site (shown in bold letters), CC (which, along with the A residue in the *Nsi*I restriction site, provided the CCA terminus of the tRNA gene), the complementary sequence of the $tRNA^{Lys,3}$ gene beginning at its 3′ end (shown in italics), the T7 promoter beginning from its 3′ end (shown as underlined), and a *Pst*I restriction site. The primer was designed as follows: 5′-GTAATACGACTCACTATAGC-3′. The double-stranded DNA contained a 3′ overhang at the 3′ end of the template; these sequences correspond to *Pst*I complementary sequences necessary for cloning in a forced orientation. The double-

stranded DNA was digested with *Eco*RI and, as an *Eco*RI–*Pst*I fragment, was inserted into the *Eco*RI and *Pst*I sites of the pSP65 vector. The pSP65 vector was purchased from Promega Corp. Large quantities of the resulting plasmid, pLys,3, were prepared by standard procedures (Sambrook *et al.*, 1989).

Synthesis of tRNA^{Lys,3} Transcript. tRNA^{Lys,3} was transcribed from plasmid pLys,3 that had been linearized with *Eco*RI. The wild-type transcript was generated with the T7 RNA polymerase transcription system (Gibco-BRL, Inc.) in the presence of α-[^{32}P]UTP, following the manufacturer's suggested protocol. Unincorporated α-[^{32}P]UTP was removed by passing the reaction mixture over a NICK column. 5′-Phosphorylation of d(T)$_{16}$ was carried out as described by Sobol *et al.* (1991).

Northwestern and Southwestern Blots. Northwestern and Southwestern blotting was performed by the method of Bowen *et al.* (1980), and blots were processed according to Lutz-Reyermuth and Keene (1989). Protein samples were applied to 15% SDS gel (Laemmli, 1970), and the proteins from gels were electrophoretically transferred onto nitrocellulose paper for 16 h at 35 V in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3 at 4 °C (Towbin *et al.*, 1979). The blot was treated with probing buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll) for 2 h at room temperature. The blot was reacted with 70 × 10^4 cpm of ^{32}P-labeled tRNA^{Lys,3} ($\simeq$0.3-μg transcript) and 6 μg of poly(A) or with 1 × 10^6 cpm of ^{32}P-labeled d(T)$_{16}$ (0.5 μg) and 54 μg poly(A) to 4 mL of probing buffer for 2 h at room temperature. The nitrocellulose paper was washed three times with probing buffer and an additional three times with probing buffer-containing 300 mM NaCl. Finally the nitrocellulose paper was air-dried and subjected to autoradiography at −70 °C with X-omat film (Kodak).

Domain Mapping. A total of 20 μg of p66, p66/p51 complex, or p51 was cleaved with V8 protease by incubation in 0.1% SDS at substrate–enzyme (S–E) ratio of 1:1 at 25 °C for 5 min or 2 h (Sobol *et al.*, 1991). The reaction was stopped by adding SDS sample buffer. Proteolytic cleavage of p51 was carried out at 25 °C by incubating p51 (0.5 mg/mL) with trypsin (20:1 or 10:1 w/w) in 100 mM NaCl and 10 mM DTT for 45 or 90 min. Reaction was stopped by adding SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptothanol, and 0.001% bromophenol blue). Samples were applied to 15% SDS gel, and the gel was stained with Coomassie blue.

Other Methods. Western blot analysis and protein microsequencing were performed as described (Sobol *et al.*, 1991). The concentration of the proteins was determined from Bradford assays (Bradford, 1976) using the Bio-Rad Protein assay kit (Bio-Rad Laboratories). Markers and their respective molecular masses were as follows: Rainbow markers (M_r 14 300–200 000); myosin, 200 kDa; phosphorylase *b*, 97.4; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa; and Rainbow markers (M_r 2350–46 000); ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.3 kDa; aprotinin, 6.5 kDa; insulin b chain, 3.4 kDa; insulin a chain, 2.35 kDa.

RESULTS

Production of RT Domain Fragments by Staphylococcus aureus V8 Protease. Controlled proteolysis of proteins often can generate domains that provide insight about structure–function relationships and the secondary structure of a protein. This approach was extended to HIV-1 RT, which was found to have a structure consistent with a very tightly folded, protease-resistant molecule. It was necessary to partially unfold the protein with denaturing agents before carrying out proteolysis. One such condition was developed by Sobol *et al.* (1991) for proteolysis of oligo d(T) cross-linked p66 by V8 protease; the condition included the presence of 0.1% SDS and 10 mM DTT.

Table I: Summary of Sequence Analysis of RT Fragments

proteolytic fragment	fragment primary structure: measured N-terminal sequence[a]	approximate C-terminus[b]	yield (pmol) at cycle 1
A. V8 Protease			
p36$_{(1\simeq300)}$	^{1}PISPIE^{6}	----------300	5
p34$_{(1\simeq290)}$	^{1}PISPI^{5}	----------290	15
p30$_{(303\simeq560)}$	E^{1303}LAENREILKE^{312}	----------560	11
p29$_{(1\simeq230)}$	^{1}PISPIETVP^{9}	----------230	15
p23$_{(123\simeq255)}$	E^{1123}DFRKYTAFTIPS^{134}	--------255	6
p18$_{(45\simeq200)}$	E^{145}GKISKIGPEN^{54}	----------200	6
p15$_{(1\simeq145)}$	^{1}PISPIE^{6}	----------145	8
p12$_{(195\simeq300)}$	E^{1195}IGQHRTKIEEL^{205}	---------300	16
B. Trypsin			
p29$_{(1\simeq230)}$	^{1}PISPI^{5}	----------230	15

[a] Measured sequences start after the numbered residue. Cleavage sites are illustrated by arrows. [b] Letters designate amino acid residues actually sequenced. Numbers correspond to the deduced sequence of HXB2 proviral DNA-deduced RT (Becerra *et al.*, 1991). The C-terminal residues shown are estimates only, from SDS–PAGE migration behavior. For example, the C-terminus of the V8 protease product p12$_{(195\simeq300)}$ is probably Glu^{291}, corresponding to the 97-residue peptide I^{195}–E^{291}. Yet, the RT sequence in this region is ^{291}EVIPLTEEAELELAERE^{308}, and any of the Glu residues could correspond to the C-terminus of p12$_{(195\simeq300)}$.

We found that upon proteolysis of p66, p66/p51 complex, or p51 with V8 protease, several domain fragments are produced (Figure 1). These domain fragments arise from N-terminal, middle, or C-terminal regions of RT, as confirmed by sequencing (Table I). For example, digestion of individual RT proteins for 5 min with V8 protease generates domain fragments that are specific for the N- and C-terminal regions of the protein (Figure 1a, lanes 3, 6, 9, and 12; Figure 1b, lanes 2, 5, and 8). Four polypeptides are detected upon SDS–PAGE, designated p36$_{(1\simeq300)}$, p34$_{(1\simeq290)}$, p30$_{(303\simeq560)}$, and p29$_{(1\simeq230)}$. Sequence analysis of these fragments from their N-termini (Table I) indicated that p36$_{(\simeq300)}$, p34$_{(1\simeq290)}$, and p29$_{(1\simeq230)}$ are extreme N-terminal fragments, starting at Pro^1, followed by 5–10 amino acid residues that match perfectly with the N-terminal region of RT. These three fragments were present in the digest of all three preparations of RT and accumulated to a similar extent; thus, as judged by the location and accessibility of V8 protease cleavage sites, these three different RT forms appear to be folded in a similar fashion.

Domain fragment p30$_{(303\simeq560)}$ represents the C-terminal end of p66; note that it was absent in the p51 digestion products. The sequence of this polypeptide begins at Leu^{303}, and the N-terminal sequence analysis of its first 10 residues demonstrated a sequence perfectly matching that of the RT sequence. Thus, the 5-min digestion condition used in this experiment is useful, as fragments of the N- and C-terminal regions of RT can be produced in large amounts. Many of the polypeptides in the range of 28–40 kDa in Figure 1a (lanes

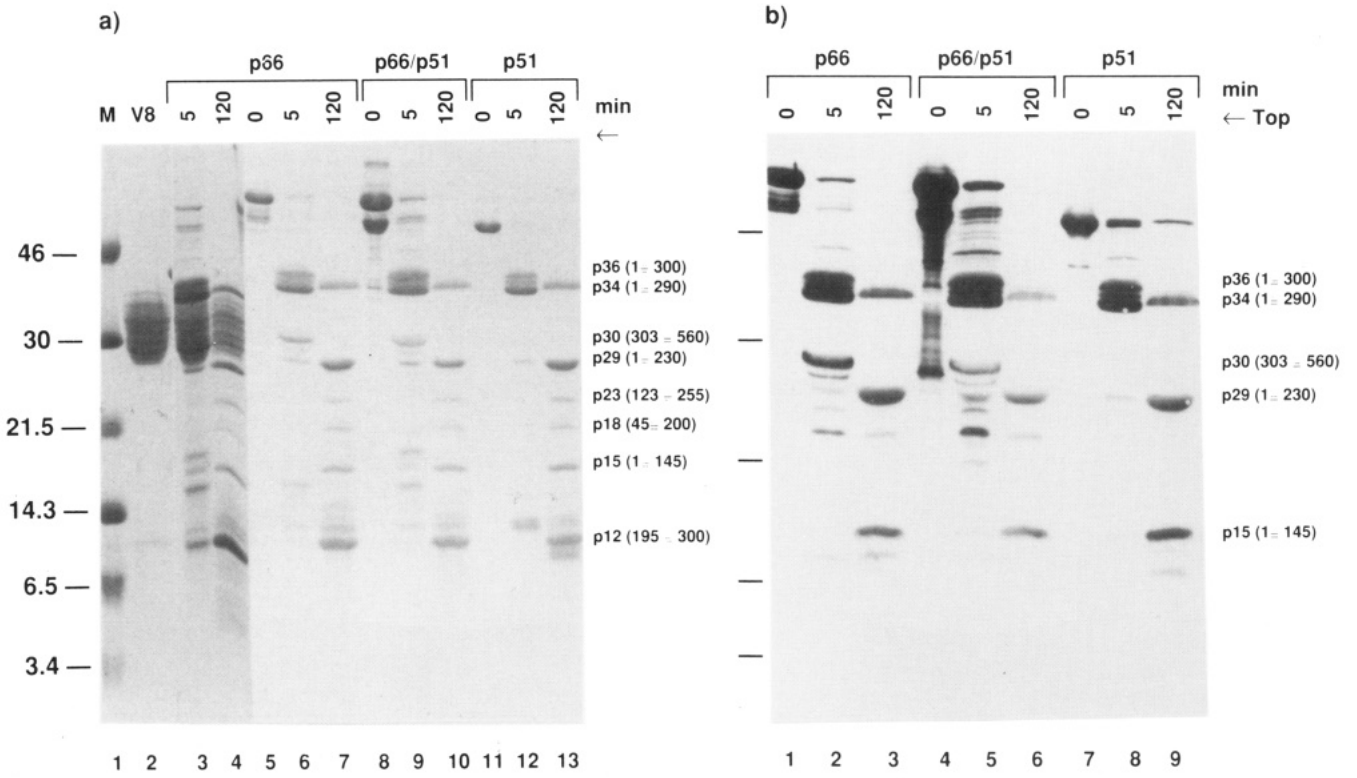
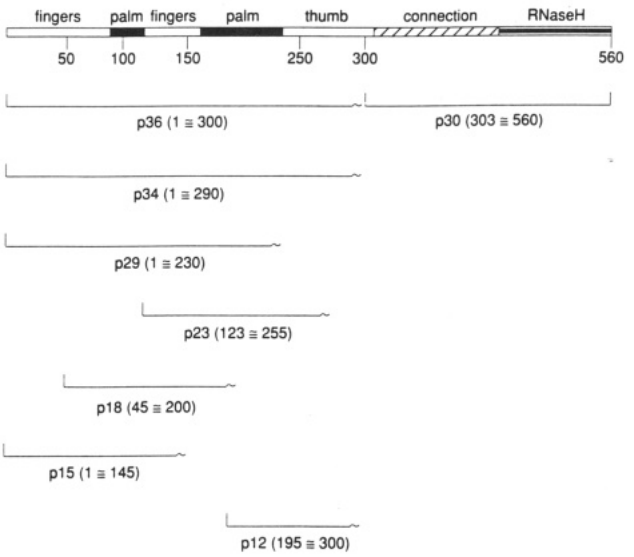


FIGURE 1: Production of domains by *Staphylococcus aureus* V8 protease and SDS–PAGE or immunoblot analysis of domain fragments. (Panel a) Photograph of a Coomassie blue-stained gel is shown. (Lanes 1–4) Gel was stained with Coomassie blue immediately after electrophoresis; (lanes 5–13) proteins were transferred electrophoretically to nitrocellulose membrane for 3 h at 60 V, then the gel was stained with Coomassie blue. Lanes 3–7 are from a digest of p66, lanes 8–10 are from a digest of p66/p51 complex, and lanes 11–13 are from a digest of p51. Each digestion was carried out with V8 protease (1:1, w/w) and was at 25 °C for 0–120 min as indicated at the top of the figure. Lane M contains Rainbow markers (M_r 2350–46 000), and lane 2 contains 20 μg of V8 protease. (Panel b) Photograph of an immunoblot probed with a mixture of monoclonal antibodies, mAb 42 (1:100 dilution) mAb NEN (1:1000 dilution), and mAb 50 (1:100 dilution) and stained with 4-chloro-1-naphthol is shown. Lanes are the same as described for lanes 5–13 in panel a. Domain fragments that were sequenced from the N-terminal end are marked on the right-hand side of panels.

2–4) represent V8 protease itself, and these polypeptides are transferred from the SDS–PAGE gel with batter efficiency than RT peptides, as seen in Figure 1a (lanes 5–13). The V8 protease peptides are not reactive with monoclonal antibodies specific for RT (Figure 1b).

When the individual RT preparations were digested for 2 h and then subjected to SDS–PAGE, seven polypeptides in the range of 12–34 kDa were seen on the Coomassie blue stained gel (Figure 1a, lanes, 4, 7, 10, and 13), whereas only three or four of these polypeptides reacted with our RT monoclonal antibody mixture (Figure 1b, lanes 3, 6, and 9). Transitions of degradations and accumulations of polypeptides can be deduced for the patterns of products by comparison of digestion periods of 5 min and 2 h. Domain fragments $p36_{(1\cong300)}$ and $p34_{(1\cong290)}$ were degraded to three fragments ($p29_{(1\cong230)}$, $p23_{(123\cong255)}$, and $p12_{(195\cong300)}$) by three separate cleavages, as confirmed by sequencing (Table I). The major products were $p29_{(1\cong230)}$ and $p12_{(195\cong300)}$, while minor cleavage occurred at Glu^{122} to generate $p23_{(123\cong255)}$. Domain fragment $p12_{(195\cong300)}$ accumulated in a large amount and remained stable for 2 h. Sequence analysis revealed that $p12_{(195\cong300)}$ starts at residue Ile^{195}, represents the middle region of RT, and is identical to the oligo d(T) cross-linked peptide found by Sobol *et al.* (1991). On the other hand, $p29_{(1\cong230)}$ degrades into two other fragments by two separate cleavages generating $p18_{(45\cong200)}$ and $p15_{(1\cong145)}$. $p15_{(1\cong145)}$ represents the N-terminal fragment, starting at Pro^1 of RT, while $p18_{(45\cong200)}$ is generated after cleavage at Glu^{44}. These fragments were not sequenced from their respective C-terminal ends. Thus, based on their mobility in SDS–PAGE, the following primary structures were assigned for the sequentially produced V8 protease digestion products [the diagram at the top is from Kohlstaedt *et al.* (1992)]:



Nucleic Acid Binding Residues in Two Discrete Regions. The Southwestern or Northwestern blotting technique had been used for qualitative detection of DNA or RNA binding to proteins after SDS–PAGE separation and subsequent electrophoretic transfer to nitrocellulose membrane [e.g., Lutz-Reyermuth and Keene (1989)]. Once the primary structure of each domain fragment was assigned, the nucleic acid-binding capacity of the RT domain fragments was examined by

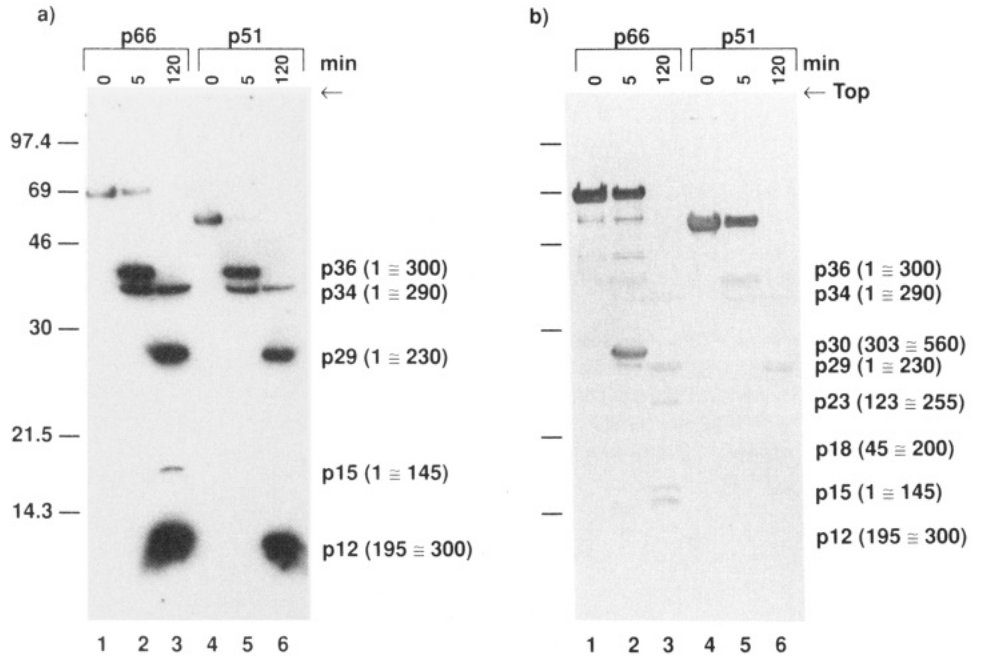


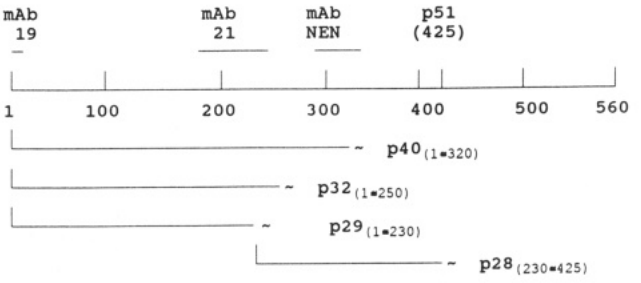
FIGURE 2: Southwestern blot of domain fragments produced by V8 protease. p66, p51, or their domain fragments (as depicted at the top of the figure) were separated by SDS–PAGE and transferred to a nitrocellulose membrane as described under Materials and Methods. The nitrocellulose membrane was then probed with ^{32}P-labeled d(T)$_{16}$. Lanes 0, 5, and 120 represent the digestion of p66 or p51 with V8 protease for 0, 5, and 120 min, respectively. (Panel a) Autoradiogram of the gel. The numbers (kDa) on the left indicate the migration of molecular weight markers. (Panel b) Photograph of an immunoblot. The presence of domain fragments on the nitrocellulose membrane used in panel a was determined by Western blotting after preparing an autoradiogram as described in Figure 1, panel b.

Southwestern and Northwestern techniques using ^{32}P-labeled primer analogues d(T)$_{16}$ or recombinant primer tRNA^{Lys,3} as a probe, respectively.

After digestion of p66 or p51 with V8 protease, the domain fragments were separated and transferred to a membrane, and the membrane was then probed with the ^{32}P-labeled primer analogue d(T)$_{16}$ (Figure 2a). Similar results were obtained when ^{32}P-labeled tRNA^{Lys,3} was used as a probe (not shown). After autoradiography, the same sheet of nitrocellulose membrane was probed with the mixture of monoclonal antibodies (mAb 42, mAb NEN, and mAb 50) to RT to precisely correlate binding with the presence of RT fragments (Figure 2b). The distribution of domain fragments as probed by monoclonal antibodies was similar to that described in Figure 1. The results indicated that domain fragments of individually purified p66 and p51 interacted with the d(T)$_{16}$ probe in a similar fashion (Figure 2a, compare lanes 2 and 3 with lanes 5 and 6). After 5-min V8 digestion, p36$_{(1\cong300)}$, p34,$_{(1\cong290)}$, and the residual undigested protein bound the probe. Also, a major fragment arising from the C-terminal end of p66 (p30$_{(303\cong560)}$) was not capable of interacting with the probe. These results indicate that all of the nucleic acid-binding activity recovered by these methods resides between residues 1 and 302.

When the p66 and p51 domain fragments generated by 2 h of digestion were assayed, no radioactive signal was observed in the region of two minor domain fragments p23$_{(123\cong255)}$ and p18$_{(45\cong200)}$, even though they fall within residues 1–300. This may be due to the fact that these fragments were minor digestion products and only a small amount was present on the nitrocellulose paper. However, three additional domain fragments showed binding capacity to the d(T)$_{16}$ probe (Figure 2a, lanes 3 or 6). These domain fragments were p29$_{(1\cong230)}$, p15$_{(1\cong145)}$, and p12$_{(195\cong300)}$. The difference in intensity of the radioactive signal represented the amount of each polypeptide present on the nitrocellulose paper as judged by sequencing (Table I). These results indicate that there are two discrete nucleic acid binding regions in RT, which reside in p29$_{(1\cong230)}$ and p12$_{(195\cong300)}$.

Tryptic Domain Production and Nucleic Acid Binding to Region I. Digestion of p51 was carried out with trypsin under nondenaturing conditions in the presence of 10 mM DTT. Domain fragments were produced at substrate–enzyme ratios of 20:1 and 10:1, and these fragments remained resistant for a period of 90 min (Figure 3a). To map the major domain fragments within the primary structure of RT, Western blot analysis was conducted using three different monoclonal antibodies (Figure 3b). There were three major bands in the range of 29–40 kDa, while some minor bands of lower molecular weight were seen also. The results indicated the following primary structures of these tryptic domain fragments:



The three major domain fragments, p40$_{(1\cong320)}$, p32$_{(1\cong250)}$, and p29$_{(1\cong230)}$, immunoreacted with mAb19, indicating that they arise from the N-terminal end of the RT polypeptide. Nucleic acid binding of these domain fragments to ^{32}P-labeled d(T)$_{16}$ or tRNA^{Lys,3} is shown in Figure 3c. The results indicated that the p40$_{(1\cong320)}$ polypeptide, which can be seen in only minor amounts by Western blotting (Figure 3c, lane 9), is not detected by the probe. p32$_{(1\cong250)}$ and p29$_{(1\cong230)}$, which are present in equal amounts as judged by Western blotting (Figure 3c, lane 9), have different nucleic acid-binding capacities, with p29$_{(1\cong230)}$ greater than p32$_{(1\cong250)}$ (Figure 3c, lanes 3 and 6). Detection of binding to p28$_{(230\cong425)}$ was

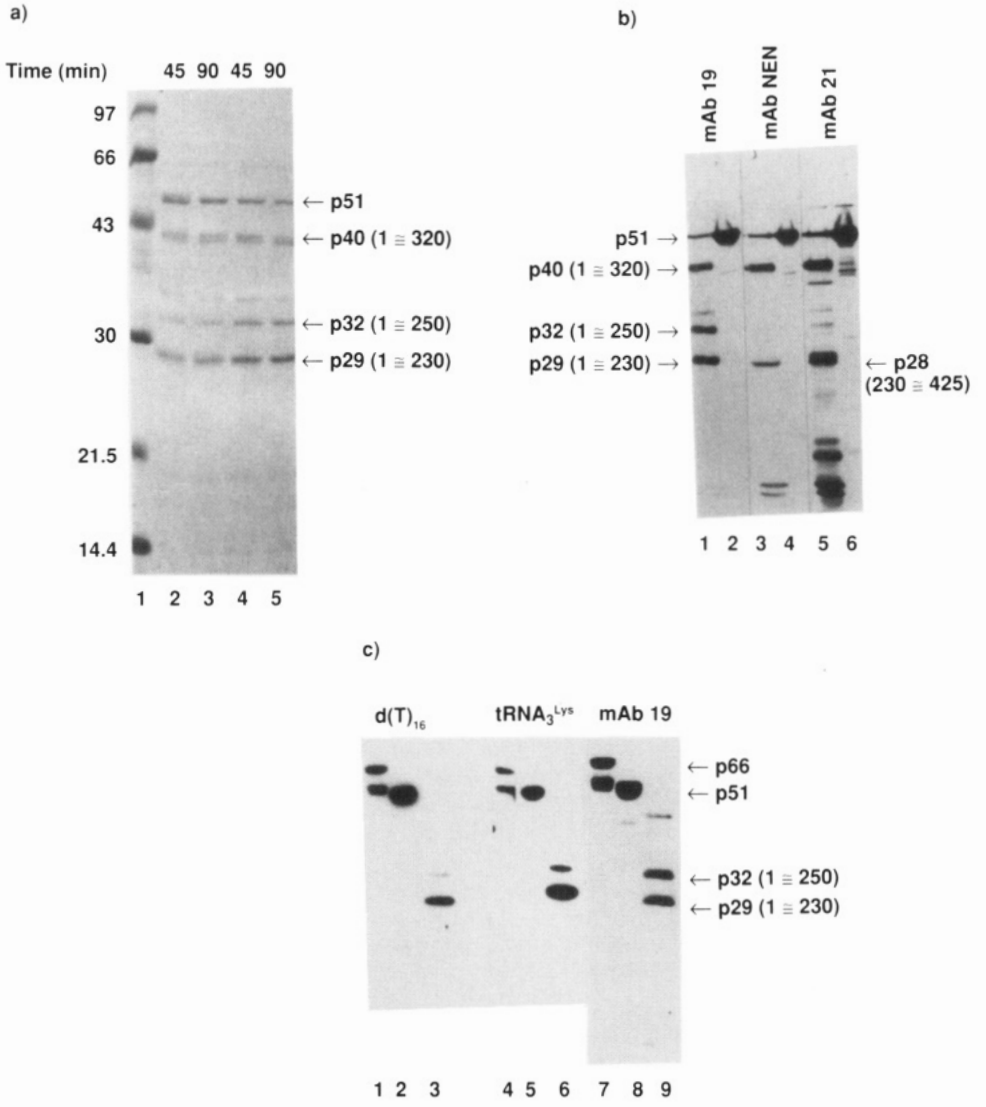


FIGURE 3: Production, mapping, and nucleic acid binding of domains generated by trypsin digestion of p51. (Panel a) Photograph of a Coomassie blue stained gel. p51 was digested with trypsin at S–E ratio of 20:1 (lanes 2 and 3) and 10:1 (lanes 3 and 4) at 25 °C for the time indicated at the top of the figure. Samples were processed as described under Materials and Methods. Positions of domain fragments are marked by arrows. Lane 1 contains the molecular weight markers. (Panel b) Photograph of an immunoblot which was obtained after Western blot analysis of domain fragments. p51 was digested with trypsin at S–E ratio of 10:1 for 90 min at 25 °C as described above, and the digest was applied in lanes 1, 3, and 5. Lanes 2, 4, and 6 each contained 3 μg of p51. Proteins were transferred to the nitrocellulose membrane electrophoretically. Nitrocellulsoe membrane strips were probed with mAb 19 (1:100 dilution), mAb NEN (1:1000 dilution), and mAb 21 (1:100 dilution) as indicated at the top of the panel. (Panel c) Southwestern or Northwestern blotting of domain fragments produced by trypsin. 3 μg of p66/p51 complex (lanes 1, 4, 7), 3 μg of p51 (lanes 2, 5, 8), and trypsin digest of p51 at S–E ratio of 10:1 for 90 min at 25 °C (lanes 3, 6, 9) were processed as described in Figure 2. Nitrocellulose membrane strips were probed as described at the top of the panel.

minimal, since it was present in only a minor amount (see Western blot Figure 3b, lanes 3 and 5). These results on nucleic acid binding by the N-terminal tryptic domain fragment $p29_{(1\cong230)}$ are in close agreement with results with V8 protease-derived fragments.

Nucleic Acid Binding by Designed RT Polypeptide Containing Region II. The V8 protease domain fragment $p30_{(303\cong560)}$ does not show binding by the Northwestern blotting assay, whereas the V8 protease or trypsin $p29_{(1\cong230)}$ domain fragment binds to nucleic acid. To further examine the importance of residues between these fragments, 231–302, we created an expression construct to extend $p30_{(303\cong560)}$ by adding 55 amino acids at the N-terminal end. The resulting expression vector (pRC-35) produces a polypeptide of 312 amino acids (residues 1-24 and 273–560). Purification of p35 was carried out using two column steps; the total extract was applied to a Q-Sepharose column, and p35 was recovered in the flow-through fraction. The flow-through fraction was applied to a Mono S column on an FPLC system. p35 was eluted with a linear salt gradient, and peak fractions containing pure p35 were pooled and used for analysis. Binding of p35 to $tRNA^{Lys,3}$ was investigated using the Northwestern blotting assay (Figure 4a,b). The results showed that p35 binds to $tRNA^{Lys,3}$ (Figure 4b, lanes 3 and 4). Thus, extending $p30_{(303\cong560)}$ at the N-terminal end to residue 273 enables this protein to bind nucleic acid, suggesting that the 30 amino acid segment of residues 273–302 contains an important site for nucleic acid–RT interaction.

In another set of experiments, to completely rule out the ability of the purified C-terminal domain to conduct nucleic acid binding, a large excess of expressed, purified $p15_{(427-560)}$ RNase H was tested (Figure 4d, lane 4). As shown in Figure 4c, lane 1, the $p15_{(427-560)}$ RNase H domain peptide failed to bind $tRNA^{Lys,3}$. All together, these results indicate that the nucleic acid-binding activity of RT resides in the N-terminal region (residue 1 ≅ 300), and there are at least two

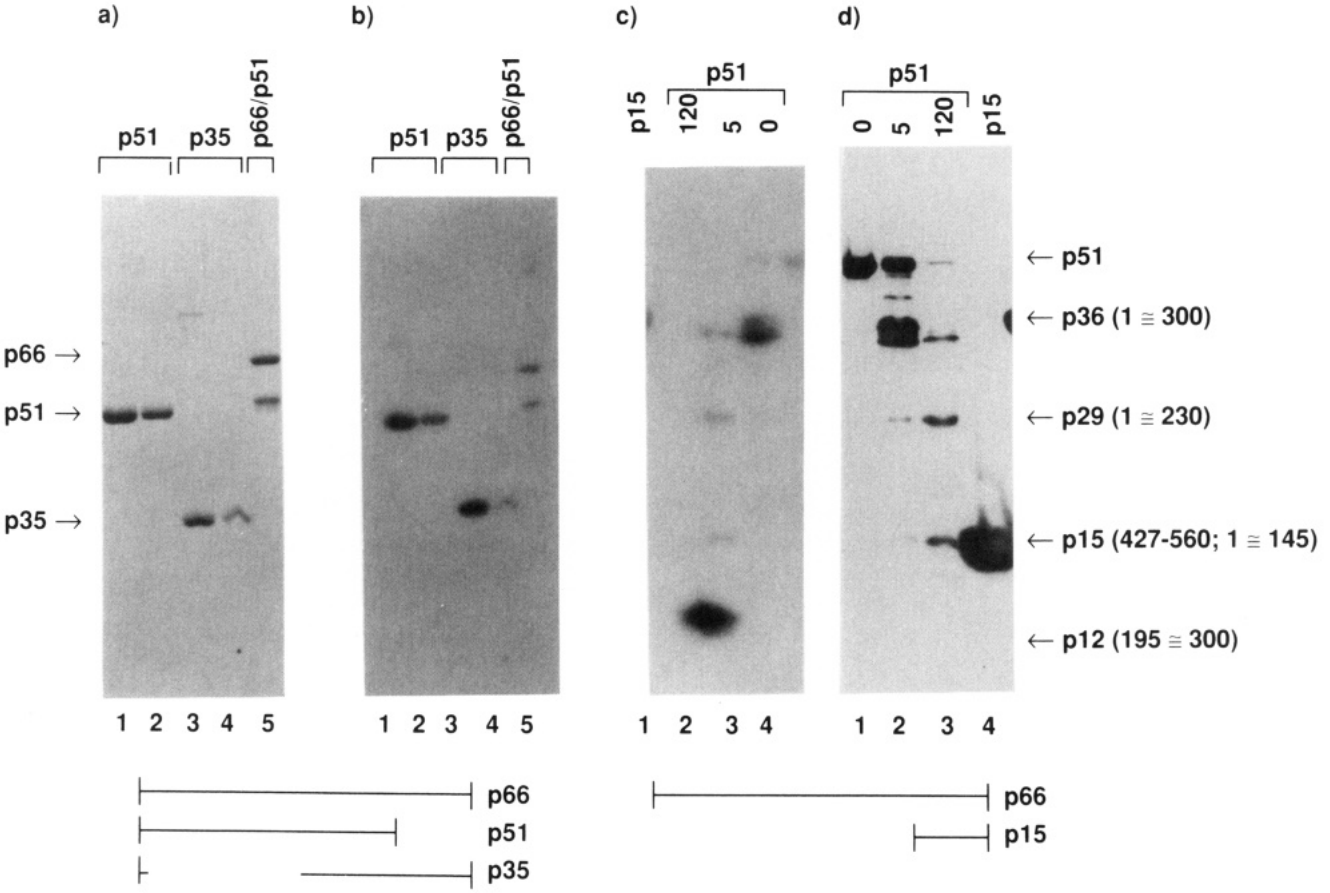


FIGURE 4: Nucleic acid binding of recombinant polypeptide of RT. (Panel a) Photograph of a Coomassie blue-stained gel. The proteins are indicated at the top of the panel. 5 and 2.5 μg of p51 (lanes 1 and 2, respectively) and of p35 (lanes 3 and 4, respectively) and 5 μg of p66/p51 complex (lane 5) were layered on the gel. (Panels b and c) Photographs of autoradiograms. Northerwestern blotting of proteins (indicated at the top) was conducted as described in Figure 2 using $tRNA^{Lys,3}$ as the probe. In panel b, the lanes are the same as described for panel a. The primary structure of the polypeptides is shown at the bottom. (Panel d) Photograph of an immunoblot probed with mAb 42, mAb NEN, and mAb 50 as described for Figure 1, panel b. The presence of $p15_{(427-560)}$ on the nitrocellulose membrane was determined by Western blotting after preparing the autoradiogram. In panels c and d, lanes 0, 5, and 120 indicate the digestion of p51 with V8 protease at S–E ratio of 1:1 at 25 °C for 0, 5, and 120 min, respectively.

nonoverlapping segments that confer nucleic acid-binding capacity termed region I (residues 1 ≅ 230) and region II (residues 273–302).

DISCUSSION

Many reports have supported the general idea that the N-terminal portion of HIV-1 RT is responsible for DNA polymerase activity, while the C-terminal end confers RNase H activity (Johnson *et al.*, 1986). However, the two domains of HIV-1 RT are clearly interdependent for function, as some mutations in the polymerase domain can affect RNase H activity, while some other mutations in the RNase H domain can disrupt polymerase activity (Hizi *et al.*, 1992, and references cited therein). Recently, the crystal structure of RT solved to 3.5-Å resolution was reported (Kohlstaedt *et al.*, 1992). The structural model indicates that the p66 subunit is folded into five subdomains: the RNase H domain and the four subdomains of the DNA polymerase (pol) domain. Three subdomains of the pol domain resemble a right hand and are named "fingers, palm, and thumb". The fourth pol subdomain connects the RNase H domain to the pol domain and is named the "connection domain". Controlled proteolysis of proteins can often generate domains which reflect the three-dimensional structure of the protein. In the present study, domain mapping of RT was carried out using two proteases, and it is interesting to consider the results in light of the structural model. Digestion of RT with V8 protease generated domain fragments which represent N-terminal, middle, and C-terminal regions of RT. The conditions for degradation were the same as those used by Sobol *et al.* (1991) for $d(T)_{16}$ cross-linked RT. At an early stage of digestion, RT is converted into two fragments, $p36_{(1\cong300)}$ and $p30_{(303\cong560)}$. Domain fragment $p36_{(1\cong300)}$ contains three of the subdomains of the pol structure, fingers, palm and thumb, while the domain fragment $p30_{(303\cong560)}$ contains the connection domain of pol and the RNase H domain. The crystal structure model appears to be generally in agreement with our proteolytic domain mapping studies. Domain fragments $p36_{(1\cong300)}$ and $p34_{(1\cong290)}$ degrade into two major fragments, $p29_{(1\cong230)}$ and $p12_{(195\cong300)}$, and a minor fragment, $p23_{(123\cong255)}$. The three-dimensional structure of domain fragment $p29_{(1\cong230)}$ also fits well with the crystal structure model where this domain contains the fingers and the palm domains of pol. $p12_{(195\cong300)}$, which covers half of the palm and most of the thumb regions, accumulates in a large amount and must have a tightly folded structure in solution. $p23_{(123\cong255)}$ covers part of the finger, palm, and thumb regions. $p29_{(1\cong230)}$ further degrades to generate $p15_{(1\cong145)}$ which covers the fingers and part of palm.

Controlled proteolysis of p66 with trypsin was studied by Lowe *et al.* (1988), and their results match very well with our findings. They showed that p66 cleaves into p66/p51, and finally p51 cleaves into p29 (residues 1-223) and p30 (residues 224 to 430–440). When our p51 was cleaved with trypsin, we observed that the first cut generated a peptide ($p40_{(1\cong320)}$)

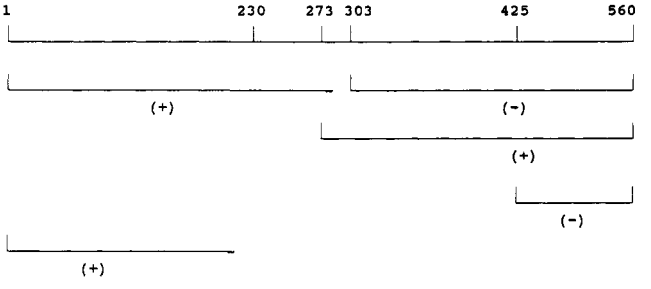
which is the domain equivalent to $p36_{(1\cong300)}$ produced by V8 digestion. $p40_{(1\cong320)}$ contains all three subdomains of pol in the structural model, in contrast to $p36_{1\cong300)}$ where about 30 residues were missing within the thumb region. $p40_{(1\cong320)}$ cleaves to $p32_{(1\cong250)}$ and finally to $p29_{(1\cong230)}$. This $p29_{(1\cong230)}$ fragment represents the same domain fragment, as was seen with V8 digestion and is similar to a fragment (residues 1–223) reported by Lowe *et al.* (1988). A small amount of domain fragment $p28_{(230\cong405)}$ is also seen on our gels, and this fragment is equivalent to p30 (^{224}HQKEPP to 430–440), as described by Lowe *et al.* (1988). Overall, the domain mapping of p66 and p51 match each other very well even though the conformation of the pol domain of p66 is different from that of p51 in the structural model of the heterodimer of p66/p51 (Kohlstaedt *et al.*, 1992). This suggests that the tertiary structures of the subdomains are similar within the p66 and p51 peptides. There are some differences that were observed in the crystal structure, mainly in the connection domain, which is not included in these domain fragments.

Retroviral RTs use host cell tRNAs as the primer for the first strand of synthesis of complementary DNA. Thus, primer recognition appears to be an important step in the viral life cycle. Many groups have reported high-affinity binding between RT and $tRNA^{Lys,3}$ or primer analogue $d(T)_{16}$ (Jacobo-Molina & Arnold, 1991; Sobol *et al.*, 1991; Basu *et al.*, 1992). We further investigated the interaction here using domain fragments produced by controlled proteolysis. Nucleic acid-binding capacity to these ligands was found to reside within the first 300 amino acids of the RT, and fragments spanning residues 303–560 failed to bind nucleic acids. Amino acids 1–300 are part of the polymerase catalytic center of RT, as proposed from the crystal structure (Kohlstaedt *et al.*, 1992), and thus, it is reasonable that nucleic acid contacts with the protein lie within this region. These findings are also in good agreement with our earlier observation that two V8 protease peptides, $p36_{(1\cong300)}$ and $p12_{(195\cong300)}$, contained the covalently cross-linked primer analogue $d(T)_{16}$ (Sobol *et al.*, 1991). It seems well-established that the central and C-terminal parts of p66 are involved in protein–protein interaction and RNase H activity (Jacobo-Molina & Arnold, 1991: Becerra *et al.*, 1991). An overexpressed RNase H domain polypeptide $p15_{(427-560)}$ does not interact with nucleic acid, confirming that most of the template–primer binding site is provided by the pol domains of RT.

The importance of the residues just N-terminal to the start of $p30_{(303\cong560)}$ was investigated by adding 55 amino acids to the N-terminal end of $p30_{(303\cong560)}$. These 55 amino acids represent two regions within the RT sequence, namely, residues 1–24 and 273–302. The p35 polypeptide bound $tRNA^{Lys,3}$, indicating that the additional 55 residues at the N-terminal end of $p30_{(303\cong560)}$ are very important for nucleic acid interaction. The first 24 residues of RT do not seem to be important for binding to nucleic acid for the following reasons: (1) comparison of the conserved regions of the polymerization domain of retroviral RTs indicates that in HIV-1 RT, these regions extend only from Gln^{23} to Trp^{266} (Jacobo-Molina & Arnold, 1991); (2) in the crystal structure these amino acids are in the finger domain and are not structured. Thus, it is likely that residues 273–302 are responsible for conferring nucleic acid binding to the otherwise binding negative 303–560 sequence. In addition to the results presented here, we note that primer analogue nucleic acid binding has not been detected for the region C-terminal to residue 303. Basu *et al.* (1992) showed that residues (288–307) in a single tryptic polypeptide are UV cross-linked to ^{32}P-labeled $d(T)_{15}$. Furthermore, Leu^{289}-Thr^{290} and Leu^{295}-Thr^{296} were identified as the probable sites of the cross-linking. Similarly, Mitchell and Cooperman (1992) mapped the active site of HIV-1 RT with phenylglyoxal and provided evidence for the involvement of Arg^{277} in primer–template binding, possibly as an essential residue. These observations point to the importance of residues within the N-terminal extended residues, 273–302, of p35. According to the crystal structure, these residues are in the thumb region (Kohlstaedt *et al.*, 1992).

To make a more detailed correlation between structure and nucleic acid binding, it is interesting to further compare our results with the crystal structure model. In the crystal structure, the fingers and the palm domains are not continuous in the primary structure, as the fingers domain covers residues $1 \cong 75$ and $120 \cong 160$ while the palm is designated by residues $75 \cong 120$ and $160 \cong 240$. Thumb, connection, and RNase H domains are represented by continuous segments (Kohlstaedt *et al.*, 1992). Domain fragments $p36_{(1\cong300)}$, $p34_{(1\cong290)}$, $p29_{(1\cong230)}$, and $p15_{(1\cong145)}$ bind to nucleic acid, and $p36_{(1\cong300)}$ and $p34_{(1\cong290)}$ contain all three subdomains of the pol domain. $p29_{(1\cong230)}$ covers the full fingers and palm domains, while $p15_{(1\cong145)}$ contains the full fingers domain. Domain fragments $p23_{(123\cong255)}$ and $p18_{(45\cong200)}$ fail to bind nucleic acid; $p23_{(123\cong255)}$ contains part of the fingers, palm, and thumb domains, and $p18_{(45\cong200)}$ represents part of the fingers and the palm domains. The explanation as to why these fragments do not bind to a radioactive probe is unclear. It is recognized that the Northwestern or Southwestern blotting assay for nucleic acid binding depends upon proper renaturation or refolding of the fragments, as well as on intrinsic ability of a renatured domain fragment to bind. Some fragments may not exhibit binding because they do not refold properly.

Further evaluation of nucleic acid binding by domain fragment $p29_{(1\cong230)}$ indicates that there is a second site in RT capable of binding nucleic acid. Thus, by use of these domain fragments, we have found a new nucleic acid-binding site which resides in the fingers subdomain and may be in contact with the template, as shown in the proposed structural model (Kohlstaedt *et al.*, 1992). Thus, two regions of nucleic acid-binding capacity are found within RT, and these are summarized below (binding-positive and binding-negative are indicated):



From these results and from the structural model, it is clear that there are multiple site(s) within RT making contact with nucleic acid. Mutations that make RT resistant to AZT or ddI (Larder & Kemp, 1989; St. Clair *et al.*, 1991) fall in the $p29_{(1\cong230)}$ proteolytic domain defined here: Met^{41}, Asp^{67}, Lys^{70}, Leu^{74}, Thr^{215}, and Lys^{219}. The approximate locations of these residues within the fingers subdomain and the palm–thumb subdomain suggest that these residues may play a role in RT template–primer interaction (Kohlstaedt *et al.*, 1992).

Clearly, much more study is required to pinpoint the amino acids involved in RT–nucleic acid interactions and the

mechanism of involvement of these amino acids in template or template–primer recognition. Based upon the results described here, we have recently overexpressed large amounts of two peptides similar to those found here, namely, p37 (residues 1–310) and p14 (residues 195–310). These peptides should help to further narrow the regions involved in protein–nucleic acid interactions (Kumar *et al.*, unpublished observations) and will also be utilized for other structural approaches, such as NMR analysis and X-ray crystallography. Such studies may help in designing inhibitors of the enzyme based upon blocking nucleic acid interactions.

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