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Subunit Interactions of Recombinant HIV-1 Reverse Transcriptase With Mutations at L289

Jacquelyn R. Smith
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Subunit Interactions Of Recombinant HIV-1 Reverse Transcriptase With Mutations At L289

by

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A Dissertation Submitted to the Faculty of Old Dominion University and Eastern Virginia Medical School in Partial Fulfillment of the Requirement for the Degree of

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ABSTRACT

Subunit Interactions Of Recombinant HIV-1 Reverse Transcriptase With Mutations At L289

Jacquelyn Ross Smith
Old Dominion University, 1994
Director: Dr. Laura K. Moen

Reverse transcriptase (RT) is a dimeric enzyme required for replication of the human immunodeficiency virus (HIV). If the subunits of the RT dimer are dissociated, the enzyme is no longer active; therefore, identification of subunit binding sites could lead to potential targets for antiviral therapy. In order to identify where subunit binding of RT occurs, mutations were made at leucine (L) 289, a residue believed to be involved in dimerization through hydrophobic interactions with other leucines. L289 is the central leucine of a leucine repeat sequence which resembles a leucine zipper protein-DNA binding motif. Two mutations, leucine to arginine (L289R) and leucine to proline (L289P), were created using PCR mutagenesis. The mutations in the RT gene were verified by DNA sequencing, and cloned into a yeast expression vector using recombinant DNA techniques. The RT mutants were purified from yeast and compared to wild type RT in terms of specific activity, subunit dissociation, and subunit association using RT polymerase activity assays, fluorescence studies, and analytical gel filtration. Only the L289P mutant showed significantly less specific activity than wild type RT, and neither mutation affected RNase H activity. Both mutants dissociated into monomers slightly more easily than wild type RT, and both mutants were slower to associate to form dimers than wild type RT. L289P dimers dissociated slightly more easily than L289R dimers and were slower to reassociate. These mutations had only moderate effects on subunit interactions.
If leucine 289 was part of a leucine zipper and directly involved in subunit binding, then both mutations would have had much greater effects on subunit interactions. Also, since RT subunit interactions are known to be hydrophobic in nature, the mutation to arginine, which is very hydrophilic, was expected to have a greater effect on dimerization than the proline mutation. However, the opposite was observed and the L289P mutation had a greater effect than the L289R mutation. These results are consistent with recent 3-dimensional computer modeling studies which indicate that leucine 289 is too far from the dimer interface to be involved in subunit binding. Leucine 289 is probably very important for maintaining secondary structure and proper folding of the enzyme, but it is not directly involved in subunit interactions.
DEDICATED WITH LOVE
TO
MOM AND DAD
ACKNOWLEDGMENTS

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INTRODUCTION

Human Immunodeficiency Virus Type-1 (HIV-1) has been identified as the causative agent of AIDS (1, 2). HIV-1 is a member of the lentivirus group of slow acting retroviruses which uses reverse transcription to replicate. The virus may take up to 12 years before causing disease (3), but when it does it is always fatal. There is no cure on the horizon and currently no effective treatment (4). The way by which HIV causes AIDS is very complicated and can occur by one or more of several direct or indirect means (3). HIV infection always results in severe immuno-suppression usually followed by opportunistic infections (5). Development of an effective treatment for HIV will require reduction in viral replication and improvements in immune function (5). The life cycle of the virus can be divided into 6 steps: 1) binding of virus to host cell membrane and release of viral core contents into cell cytoplasm, 2) reverse transcription, 3) migration of viral DNA to nucleus and integration with host cell's DNA, 4) viral transcription, 5) viral protein synthesis and processing, 6) virion assembly, release of virus from cell, and maturation (6). Each of these steps are being investigated as targets for HIV drug therapy (Figure 1).

Reverse transcriptase (RT) is an enzyme required for reproduction of HIV. RT uses three different functional activities to convert the single-stranded RNA genome into double-stranded DNA so that it can be incorporated into the host cells genome (7). The first function uses RNA-dependent DNA polymerase activity to copy the single, plus-stranded genomic RNA into minus-stranded DNA using cellular tRNA Lys as a primer. The second function uses ribonuclease hybrid (RNase H) activity to degrade the viral RNA template bound to the newly synthesized DNA except for a small piece of the viral RNA
Figure 1. Diagram showing the main steps in the life cycle of HIV-1 which are potential targets for HIV drug therapy.
1. Binding of virus and release of core contents

2. Reverse transcription

3. Migration of provirus to nucleus and integration with host cell’s DNA

4. Viral transcription

5. Protein synthesis and processing

6. Virion assembly, release from host cell, and maturation
which will act as primer for the third step of reverse transcription. This third activity, and 
the final step of reverse transcription, is DNA-dependent DNA polymerase activity which 
synthesizes the complimentary, plus-strand of viral DNA. The viral genomic material is 
now double stranded DNA which can be incorporated into the host cell genome where it 
will be transcribed along with the host's own DNA (7). These three steps require very 
different and specific activities which are carried out by two different but interdependent 
functional domains, the polymerase domain and the RNase H domain (8, 9, 10, 11). 
Reverse transcriptase has been studied extensively since the enzyme was discovered in 
1970 (12, 13); and since HIV was identified as the causative agent of AIDS, HIV-1 RT 
has been the most thoroughly characterized of all known types of RT. However, we still 
do not know enough to design an effective drug to specifically inhibit this enzyme.

Since RT is the only enzyme required for reverse transcription, which is an 
essential step in the life cycle of this virus, RT is a primary target for anti-viral drug therapy 
(14). Currently there are only three FDA approved drugs for the treatment of AIDS and all 
three are nucleoside analogs which inhibit RT by chain termination through competitive 
inhibition of the dNTP substrate (14). These analogs are 3'-azidothymidine (AZT), 2',3' 
dideoxyinosine (ddI), and 2',3' dideoxycytidine (ddC). These drugs have various toxic 
side effects because they are not specific enough and will inhibit host cell DNA 
polymerases as well as HIV-1 RT (15). There are several other nucleoside analogs which 
are currently undergoing clinical trials including d4T, 3TC, 3' fluoro-thymidine (FLT), 
and 9-(2-Phosphonomethoxyethyl)-adenine (PMEA) (5). There are non-nucleoside analog 
inhibitors of HIV-1 RT such as the tetrahydroimidazo-[4,5,1]-[1,4]benzodiazepin-2-(1H)- 
one and thione (TIBO) derivatives (15), nevirapine, pyridones, and more (5). These 
substances appear to be very specific for HIV-1 RT and may involve an allosteric binding 
site; however, neither the mechanism of inhibition nor possible side effects are known 
(15). Another problem encountered with RT inhibitors is the formation of drug-resistant 
variants of HIV. HIV shows extreme hyper-mutability due to the high error rate of HIV-1
RT (16). Consequently, mutants arise which are no longer susceptible to most drug inhibitors. Current treatments and therapies in development have been reviewed recently (5). The more we know about the structure and function of HIV-1 RT, including knowledge of conserved and essential sequences of each domain, the more likely we will be able to design safe and effective drugs to inhibit this deadly virus. One aspect of the structure of RT that is essential for activation of the enzyme is dimerization. If we can determine where these subunit interactions take place then we can design a method for blocking these binding sites which would prevent dimerization and inhibit activity of the enzyme.

RT is a product of the polymerase (pol) gene of HIV which also codes for protease (5'end) and integrase (3'end) (17) as shown in Figure 2. RT is expressed as a 560 amino acid polypeptide and has an apparent molecular weight of 66,000; however, RT purified from virions is a dimer consisting of two subunits, a 66kD subunit (p66) and a 51kD subunit (p51), which have the same N-terminal sequences (18,19). The smaller subunit, p51 is derived from the larger p66 subunit through proteolytic processing by HIV-1 protease at a specific site, Phe 440 (20, 21). The C-terminal region cleaved from p66 contains the RNase H domain so p51 does not have any RNase H activity (22). The polymerase domain is located in the N-terminal region of both p66 and p51 (9, 23) (see Figure 3), but only the polymerase domain of the p66 subunit appears to be catalytically active (24, 25). It was found that, although recombinant HIV-RT does not contain viral protease, it still produces equimolar amounts of p66 and p51, indicating that proteolytic processing of RT can also be carried out by host cell proteases (26). By including protease inhibitors in the purification procedure researchers can obtain uncleaved p66 in equilibrium between monomer and homodimer forms (20). Even though the heterodimer p66/p51 has only one catalytically active polymerase domain, it is still the most active form of the enzyme (27). Recent research suggests that the p51 subunit serves to increase the processivity of p66/p51 (28). Increased enzyme processivity may be due to either stronger...
Figure 2. Schematic diagram showing that the three HIV-1 enzymes: protease, reverse transcriptase (RT), and integrase come from the pol gene of HIV-1. RT is made up of three domains: the polymerase domain, the tether domain, and the RNase H domain.
**GAG**  **POL**  **ENV**

**Protease**  **RT**  **Integrase**

**Polymerase**  **Tether**  **RNase H**
Figure 3. Schematic diagram of HIV-1 reverse transcriptase showing the domain arrangement and protease cleavage site which forms the p51 subunit by cleavage near the C-terminus of p66.
amino acid #440
HIV-1 protease cleavage site

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primer-template binding or better conformation of the enzyme active site. As a result of the increased processivity, p66/p51 has twice the specific activity as p66/p66 with only half as many catalytically active polymerase domains (28).

Researchers have observed an apparent connection between dimer stability or dimerization and enzyme activity (20, 27, 29, 30, 31). Dimer stability can be defined as the percentage of dimeric enzyme versus monomeric enzyme. Dimerization has been analyzed using HPLC gel filtration, analytical ultracentrifugation, polymerase activity, and fluorescence. It was found that the subunit species involved, either pure p66 or pure p51, or a mixture of p66 and p51, determines how stable the dimer is. Enzyme activity studies show that p66/p51 is the most stable dimeric form of the enzyme and has the greatest amount of activity (20). Homodimer, p66/p66, is less stable and has 20% less enzyme activity than heterodimer. Homodimer, p51/p51, has 10-20% less activity than p66/p66 (20). Accurate determination of the dissociation constant for heterodimer has been difficult and is usually achieved by either of two methods. The subunits of the heterodimer can be dissociated using 17% acetonitrile without causing them to unfold. When the concentration of acetonitrile is diluted ten-fold re-association of subunits and restoration of enzyme activity is observed (29). Another technique is to use recombinant p66 and p51 that are separately expressed and purified before combining them to observe association. Homodimer, p66/p66, is less stable than p66/p51 and appears to exist in a monomer-dimer equilibrium (27). Restle and coworkers (29) used HPLC gel filtration to study reassociation of subunits which were first dissociated with 17% acetonitrile and found the $K_a$ of purified recombinant p66 to be $2.3 \times 10^5$ M$^{-1}$. They estimated the $K_a$ of p66/p51 to be at least $10^9$ M$^{-1}$ (29). Three years later the same lab, using intrinsic protein fluorescence, determined $K_d$ of the heterodimer to be $4 \times 10^{10}$ M$^{-1}$ (32). Using analytical ultracentrifugation, Becerra and coworkers (33) determined $K_a$ for p66 to be $5.1 \times 10^4$ M$^{-1}$ and for p66/p51 heterodimer to be significantly greater at $4.9 \times 10^5$ M$^{-1}$, which is much less than what Restle and coworkers (29) determined. The difference in results may be due...
not only to the fact that two different methods were used for measuring dimerization, but also because the RT forms used were obtained by different methods and the p51 subunit was slightly different in each case. Becerra's group used separately expressed cloned p66 and p51, whereas Restle's group used p51 derived from p66 by proteolytic cleavage forming the C-terminus found in vivo. Restle's construct turned out to be 15 residues longer than Becerra's cloned p51. The p51/p51 homodimer, formed when using a cloned recombinant gene coding for the 51 kD subunit, is very unstable and rapidly dissociates into monomers (20, 29, 30). Restle and coworkers determined the $K_a$ for p51 to be $1.5 \times 10^3 \text{ M}^{-1}$ (29). All of these kinetic studies on the dimerization of HIV RT demonstrate that 1) the form of enzyme which has the greatest polymerase activity, p66/p51, also forms the most stable dimers, 2) the form of enzyme which has the least polymerase activity, purified recombinant p51, forms the least stable dimers, 3) purified recombinant p66 forms dimers that are less stable than p66/p51 and have less activity, and 4) p66 forms homodimers that are more stable than p51 homodimers and have greater enzyme activity.

Anderson and Coleman (34) correlated changes in enzyme activity with changes in conformation observed when p66 is cleaved to form p66/p51 using circular dichroism. They observed such a big change in conformation that it indicates not only the formation of a new species, p51, but also that the remaining uncleaved p66 subunit also undergoes conformational change. These observations support the theory that, in vivo, immediately after p66 is formed it associates into homodimers which are the target of HIV protease. As soon as one of the subunits is cleaved to form p51 the other p66 subunit folds, blocking the protease cleavage site resulting in only the p66/p51 heterodimer (35). The Anderson and Coleman research (34) also supports the theory that half the polymerase active sites are inactivated by formation of heterodimer but that this is offset by a much greater increase in binding affinity of heterodimer for primer-template.

Some information on the nature of the subunit interactions involved in dimerization has been obtained. Subunit interactions appear to be noncovalent and hydrophobic, based
on the behavior of the enzyme as a function of salt concentration and enzyme concentration (27, 29, 33, 36). Dimer stability and enzyme activity are favored in high salt buffers containing, for example, either 1M ammonium phosphate (27), 60mM sodium pyrophosphate (27), or 200mM sodium chloride (33). These results suggest that the subunit interactions involve hydrophobic effects because such high ionic strength solutions would inhibit ionic interactions and enhance hydrophobic effects. The concentration of RT in a sample also affects dimer stability and RT activity. Restle and coworkers (29) found that in a dilute solution homodimer (p66/p66 or p51/p51) dissociates relatively rapidly into monomers. When samples containing primarily monomers were concentrated, they showed an increase in the dimer to monomer ratio and an increase in enzyme activity (29). Similar results were obtained by Deibel and coworkers (36) who found that p66 isolated under dilute concentrations (less than 0.5 mg/ml) was primarily monomeric and had low activity, but when the p66 solution was concentrated it showed increased activity.

The presence of template-primer substrate in the enzyme solution also appears to affect enzyme stability. Rowley and coworkers (27) observed that when template-primer was added to a solution of p66, the activity of the enzyme was stabilized and did not decrease as it did in an enzyme solution which did not contain template-primer. The Divita study using fluorescence also observed an increase in dimer stability when template-primer was added to the enzyme solution (32). These results indicate that dimerization is maintained through the formation of a dimer/template-primer complex in which both of the subunits of the dimer bind to the template-primer. Several researchers support the theory that a hydrophobic dimer interface forms part of a template-primer binding site (35, 33, 37). Studies using fluorescence to observe dimer association and dissociation showed a 25% increase in tryptophan fluorescence when dimers dissociate into monomers. These researchers suggested that the tryptophans (7 in region 398 to 414) are highly hydrophobic and may be directly involved in the protein-protein interactions of dimerization (32, 38).

Mg$^{2+}$ has also been shown to be involved in subunit interactions by increasing the
association rate by 100 fold (32). Divita and coworkers (32) suggest the Mg$^{2+}$ may either induce a conformational change which supports dimerization, or may be directly involved in protein-protein interactions. There are two metal binding sites in a region of the RNase H domain which has previously been shown to be involved in protein-protein interactions (39).

It is still not known which specific amino acids participate in subunit binding. The relationship between subunit species and dimer stability suggest that one RNase H domain, as is found in p66/p51, is needed for subunit binding because p51/p51 which has no RNase H domains is very unstable; but that two RNase H domains, as is found in p66/p66, hinders subunit binding. Becerra and coworkers (33) used immunoprecipitation to analyze binding between p66 and different p66 polypeptide segments corresponding to p29 (N-terminus), p15 (C-terminus), and p51. They found that neither p29 nor p15 showed any binding to p66; only p51 bound to p66, indicating that the central region of p66, residues 252-428, is required for subunit binding (33). A recent study on the effects of certain monoclonal antibodies on heterodimer formation found that three regions are involved in subunit interactions: 1) amino acids 230-300, 2) amino acids 350-428, and 3) residues around amino acid 540 (39). Baillon and coworkers (1991), analyzing the amino acid sequence of RT in the central region, found a leucine repeat sequence which resembled the leucine zipper motif found in certain DNA binding proteins. Site-directed mutagenesis of the leucines was used to determine if they were involved in dimerization (40). This research was being carried out at the same time as my own research in which I also mutated one of the leucine residues in this region, so their results will be compared to my results in the discussion section of this paper. Briefly, they found that L289K-P66 was unable to form dimers with itself or with wild-type p66, or with L289K-p51. They believe that the leucine repeat motif in the p66 subunit is critical to formation of the heterodimer. However, wild type p66 could dimerize with mutant L289K-p51 suggesting that this residue in the p51 subunit is not at the hydrophobic protein-protein interface and is therefore not
critical to formation of the heterodimer (40).

In June of 1992 the crystal structure of HIV-1 RT complexed with the drug Nevirapine was published (41). The researchers found that the polymerase domain of p66 subunit is analogous to the Klenow fragment of *E. coli* DNA polymerase I. However, the p51 subunit, which has the identical sequence of p66 minus the RNase H domain, has a very different tertiary structure. It has been shown through research that p51 does not appear to be involved in the catalytic function of polymerization (24, 25), but it has been postulated to be necessary for template-primer binding (41) and several researchers have observed that p66/p51 has a greater template-primer binding affinity than p66/p66. The crystal structure shows that p66 has a large cleft, which p51 does not have, and it is this cleft which is believed to be the catalytic site (41). Each subunit is divided into four subdomains: 1) fingers, 2) palm, 3) thumb, which are named for their orientation in p66 relative to a right hand, and 4) a connection subdomain which corresponds to what was previously called the tether domain. The crystal structure shows that the tip of the fingers subdomain of p51 is adjacent to the palm subdomain of p66. It also shows that both of the connection subdomains of p66 and p51 are closely associated, and the thumb of p51 contacts the RNase H domain of p66 (41). Which of these associations is necessary for subunit binding and/or template-primer binding is not known.

The work in this study correlates specific changes in the amino acid sequence of RT with changes in subunit interactions and enzyme function. Before using site-directed mutagenesis to analyze structure-function relationships involved in the dimerization of HIV-1 RT, the amino acid sequences of different RT clones were compared to identify conserved residues. The RT amino acid sequences of the three original HIV-1 isolates (42, 43, 44), as well as several sequences obtained from NIH's GenBank, were compared. These studies showed that 30 out of the 560 amino acids of HIV-1 RT were variable among different RT clones, which is not surprising since it is known to be a highly mutable protein (45). The sequence comparison studies also showed that the amino acids...
in the leucine repeat region were highly conserved. Since this leucine repeat sequence is located in one of the regions identified as a possible subunit binding site (33, 39), and since it resembles a leucine zipper which involves hydrophobic subunit interactions, it will be the target of mutagensis for this research. These changes, when interpreted with the recently available crystal data and activity analyses, will identify where subunit interactions take place. This information will contribute to the overall understanding of HIV-1 RT structure and function and will provide a possible target for drug design efforts to combat AIDS and possibly other retroviral diseases.
MATERIALS

Yeast and Bacteria Cultures

*Saccharomyces cerevisiae* strain AB110, Dr. Moen's -70°C stock cultures

*Saccharomyces cerevisiae* strain XS95-6C, from U.C. Berkely Yeast Genetic Stock Center

*Escherichia coli* (E. coli) strain HB101, Dr. Moen's -70°C stock cultures

Plasmid

Yeast plasmid expression vector containing gene for wild type reverse transcriptase, pAB24/RT5, cloned in yeast strain AB110, Dr. Moen's -70°C stock cultures

Chemicals

Acetonitrile - Fisher

Acrylamide, electrophoresis grade - Fisher

Affi-Gel Blue 100-200 Mesh (wet) 75-150 µ- BioRad

Agar, Bacto - Difco

Agarose, Seakem LE, GTG, and NuSieve - FMC BioProducts

Albumin Standard - Pierce

Amino Acids - Sigma

Ammonium Persulfate (APS) - Fisher

Ammonium Sulfate - Fisher
Ampicillin, Sodium Salt - Sigma
AmpliWax PCR Gem100 pellets - Perkin Elmer Cetus
BisAcrylamide, electrophoresis grade - Fisher
Bovine Serum Albumin (BSA), nuclease free - Promega
Bromophenol Blue - BioRad
1-Butanol - Fisher
Calcium Chloride (CaCl₂), Dihydrate - Mallinckrodt
Cesium Chloride - Stratagene
Chloroform - EM Science
Coomassie Brilliant Blue R-250 - BioRad
Coomassie Plus Protein Assay Reagent - Pierce
Dextrose, Bacto - Difco
Ethylene diamine tetraacetate-2H₂O (EDTA) - Fisher
Dithiothreitol (DTT) electrophoresis grade - Fisher
Ethidium Bromide (EtBr) - ICN Biochemical
Ethanol (ETOH), 100% - AA PER Alcohol and Chemical
ETOH, 95% - McCormick Distilling
Ficoll 400 - Sigma
Glacial Acetic Acid - Fisher
Glycerol, enzyme grade - Fisher
Glycine, tissue culture grade - Fisher
Hydrochloric Acid (HCl) - EM Science
Isopropanol - EM Science
Isoamyl alcohol - EM Science
β-Mercaptoethanol (βME), electrophoresis grade - Fisher
Magnesium chloride (MgCl₂), Hexahydrate - EM Science
Methanol, HPLC grade - Fisher
P11 cellulose phosphate - Whatman
Phenylmethanesulfonyl Fluoride (PMSF) - Sigma
PEG3350 - ICN Biochemical
Peptone, Bacto - Difco
Phenol - Fisher
Potassium Chloride (KCl) - Mallinckrodt
Protein Standards: Gel Filtration Standards - BioRad
SDS-PAGE Standards - BioRad
Processing Chemicals, Kodak GBX Developer and Replenisher - Sigma
Processing Chemicals, Kodak GBX Fixative and Replenisher - Sigma
Rainex - Unelko
Scintillation Cocktail, CytoScint - ICN Biochemical
Sigmacote - Sigma
Sodium Acetate - Aldrich
Sodium Azide - Sigma
Sodium Chloride (NaCl) - EM Science
Sodium Dodecyl Sulfate (SDS) - Fisher
Sodium Hydroxide (NaOH) - Mallinckrodt
Sodium Pyrophosphate - Fisher
Sorbitol - Fisher
TEMED - BioRad
Trichloro-acetic acid (TCA) - Fisher
Tris Base - Fisher
Triton X-100 - Boehringer Mannheim
Tryptone, Bacto - Difco
Uracil - Sigma
Urea - Stratagene
Xylene Cyanole FF, electrophoresis grade - BioRad
Yeast Extract, Bacto - Difco
Yeast Nitrogen Base w/o Amino Acids - Difco
YPD powder - Difco

Enzymes
AMV-RT (8,000 U/ml) - Promega
β-Glucuronidase, 1,000,000 U dry powder (glusulase) - DuPont
Ribonuclease (RNase), deoxyribonuclease (DNase)-free pancreatic (10mg/ml) - Sigma
BamH-1 - Promega
Sal-1 - Promega
Hind-3 - Promega
Pvu-2 - Promega
AlwN-1 - New England BioLabs
Restriction Enzyme Buffers B, C, D - Promega
Pfu DNA polymerase - Stratagene
pfu Buffer 10X - Stratagene
Ligase and Ligase Buffer - Promega

Nucleotides
Lambda DNA Bst Digest Molecular Weight Marker - Sigma
Synthetic oligonucleotide primers, reconstituted to 50µM concentrations - Midland Certified Reagent Company
2’- Deoxyadenosine-5’-Triphosphate (dATP), 10mM - Sigma
2’- Deoxycytidine-5’-Triphosphate (dCTP), 10mM - Sigma
2’- Deoxyguanosine-5’-Triphosphate (dGTP), 10mM - Sigma
2’- Deoxycytidylidine-5’-Triphosphate (dTTP), 10mM - Sigma
[Methyl, 1', 2', - ^3H] -Thymidine-5-Triphosphate ([^3H]-dTTP), 1.0 mCi/ml - Amersham
(rA)-(dT)$_{12-18}$ Template-Primer - Pharmacia
poly(dT) - Pharmacia
poly [8-^3H] (rA) - Amersham

**Kits**
GeneClean - Bio101, Inc.
Silver Sequence - Promega

**Media, Buffers, Solutions**  (All solutions made with milli-Q H$_2$O and stored at room
temperature unless otherwise noted)

**YPD Medium, 1 liter:** Mix and autoclave 20gms peptone, 10gms yeast extract, and
920mls H$_2$O. Add 50mls of separately autoclaved 20% dextrose, 25mls of filter
sterilized 0.2% adenine, and 5mls of filter sterilized 1% tryptophan.

**Leucine minus rich medium, 1 liter:** Combine 10 mls Yeast Nitrogen Base (10X), 0.5 g
leucine minus amino acid supplement (0.8g adenine, 0.6g tyrosine, 0.6g lysine,
0.6g uridine, 1.0g phenylalanine, 0.4g tryptophan, 0.4g methionine, 0.4g arginine,
0.4g histidine) 5mls 1%tryptophan, 25mls 0.2% adenine, 8mls 5% threonine,
400mls 20% glucose and H$_2$O to 1 liter. Filter sterilize.

**Leucine minus selective medium, 1 liter:** first mix and autoclave 5g ammonium sulfate,
1.7g yeast nitrogen base, 20g dextrose, 1g YPD powder, 600ml H$_2$O. In a
separate container mix and filter sterilize 0.088g tryptophan, 0.127g adenine, 0.4g
threonine, 0.058g tyrosine, 0.058g lysine, 0.058g uridine, 0.096g phenylalanine,
0.038g methionine, 0.038g arginine, 0.038g histidine, 0.1g aspartic acid, 0.1g
glutamic acid, 0.39g serine, 0.155g valine, and 0.02g uracil and 400mls of H$_2$O.
Once the autoclaved portion has cooled slightly, add the filtered portion and swirl
to mix.
Leucine minus selective regeneration agar, 500mls: First mix and autoclave 2.5g ammonium sulfate, 0.85g yeast nitrogen base, 10g dextrose, 250mls 2M sorbitol, 0.5g YPD powder, 15g agar 50ml H₂O. In a separate container mix and filter sterilize 0.044g tryptophan, 0.0635g adenine, 0.2g threonine, 0.029g tyrosine, 0.029g lysine, 0.029g uridine, 0.048g phenylalanine, 0.019g methionine, 0.019g arginine, 0.019g histidine, 0.05g aspartic acid, 0.05g glutamic acid, 0.195g serine, 0.0775g valine, and 0.01g uracil and 190mls of H₂O. Once the autoclaved portion has cooled slightly, add the filtered portion and swirl to mix.

LB Medium, 1 liter: Combine 10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl, and H₂O to 1 liter. Autoclave 15 minutes.

LB selective medium, 1 liter: same as above except add 4ml of filter sterilized ampicillin (25mg/ml) when broth has cooled.

For solid media add 20g agar to 1 liter of liquid medium before autoclaving, pour plates before medium solidifies.

Yeast Breaking Buffer, for plasmid prep: 2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-Cl pH 8.0, 1 mM EDTA

Disruption Buffer for mini-multiple protein preps: 20mM Tris-Cl pH 7.9, 5% glycerol, 10mM MgCl₂, 1mM EDTA, 1mM DTT, 0.3M ammonium sulfate, 1mM PMSF

Lysis Buffer for large protein prep: 50 mM Tris-Cl pH 7.5, 20% glycerol, 100mM KCl, 0.1% Triton-X 100, 4mM EDTA, 2 mMβ mercaptoethanol, 1mM PMSF

Reverse Transcriptase Buffer (RTB): 50 mM Tris-Cl pH 7.5, 20% glycerol, 50mM KCl, 0.1% Triton-X 100, 4mM EDTA, 2 mMβ mercaptoethanol (add 1mM PMSF during purification)

RT Activity Assay Buffer (10X): 1M Tris pH8.5, 1M MgCl₂, 4MKCl, 1M DTT, 10% Triton-X 100

RT Activity Assay Stop Solution: 10% TCA, 10mM Sodium Pyrophosphate

RT Enzyme Diluent: 20μl 10X RT Activity Assay Buffer, 2μl BSA (10mg/ml), and
178μl H₂O

β-Glucuronidase: add 11.11mls H₂O to 1,000,000U powder to make 90,000 U/ml final concentration, store at -20°C

1M sorbitol, autoclave

2M sorbitol, autoclave

CaCl₂ solution: 0.1M Tris-Cl, pH7.4 and 0.1M CaCl₂, autoclave

Sorbitol/CaCl₂ solution: 1M sorbitol, 10mMTris-Cl pH7.4, 10mM CaCl₂, autoclave

PEG/CaCl₂ solution: 45% PEG3350, 10mMTris-Cl pH7.4, 10mM CaCl₂, filter sterilize

1M Tris-Cl pH 8.0

1M Tris-Cl pH 7.5

TE Buffer pH 8.0: 10mM Tris-Cl (pH 8.0), 1mM EDTA (pH 8.0)

TAE Buffer (50X): 2M Tris- Acetate, 50mM EDTA

TBE Buffer (10X, 1 liter): Combine 108g Tris Base, 55g Boric Acid, 40ml 0.5M EDTA pH8.0, and add H₂O to 1 liter

Ethidium Bromide: 10mg/ml stock solution, stored at 4°C, wrapped in foil

Phenol buffered with 0.1 M Tris-Cl pH 8.0, stored in dark bottle at 4°C

Chloroform: Isoamyl Alcohol mixed 24:1

Phenol:Chloroform: Isoamyl Alcohol mixed 25:24:1

3M Sodium Acetate, pH adjusted to 5.2 with glacial acetic acid

70% ETOH, stored at -20°C

Plasmid prep solution I: 25mM Tris-Cl pH 8.0, 50mM glucose, 10mM EDTA

Plasmid prep solution II: 0.2N NaOH, 1% SDS

Plasmid prep solution III: 3M potassium acetate, 11.5% glacial acetic acid

DNA sample loading bufer: 20% ficoll 400, 0.1 M EDTA pH 8.0, 1.0% SDS, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol

DNA Standard Molecular Weight Marker: Make a stock solution by adding 10μl of Lambda BstE-2 Digest (730μg/ml) to 40μl of DNA sample buffer, store at -20°C.
Load 3.5μl into a small well for agarose gel electrophoresis

Protein sample buffer: 62.5 mM Tris-Cl pH 6.8, 12.5% Glycerol, 1.25% SDS, 0.35M βME, 0.05% Bromophenol Blue

10% Polyacrylamide Separating Gel: 12.1 ml H₂O, 7.5 ml of 1.5 M Tris pH 8.8, 0.3 ml of 10% SDS, 10 ml of Acrylamide/Bis acrylamide (30/0.8), 0.1 ml 10% APS, and 7.5μl TEMED

3% Polyacrylamide Stacking Gel: 6.3 ml H₂O, 2.5 ml of 0.5 M Tris pH 6.8, 0.1 ml of 10% SDS, 1 ml of Acrylamide/Bis acrylamide (30%/0.8%), 0.1 ml 10% APS, and 5.0μl TEMED

SDS-PAGE Gel Stain: dissolve 1.25 g Coomassie Brilliant Blue in 227 ml methanol, add 46 ml glacial acetic acid and 227 ml H₂O, filter

SDS-PAGE Gel Running Buffer (10X): combine 60 g Tris base, 288 g glycine, and 20 g SDS, add H₂O to 2 liters

SDS-PAGE Gel Destain: combine 454 ml methanol, 92 ml acetic acid, and 454 ml H₂O

500μM dNTP mix: combine 50 μl of 10 mM dATP, 50 μl of 10 mM dTTP, 50 μl of 10 mM dGTP, 50 μl of 10 mM dCTP, and 800 μl of sterile H₂O

Superose 12 buffer: 100 mM Tris pH 8.5, 50 mM KCl, 5% glycerol, 10 mM BME, 1 mM EDTA, 0.1% TX-100

TSK buffer for protein purification: 50 mM Tris-Cl pH 7.5, 50 mM KCl, 0.1 mM DTT, 0.2 mM EDTA, and 0.1% TX-100

6% DNA Sequencing Gel: Dissolve 25.2 g Urea in 24 ml H₂O, add 6 ml 10X TBE and 9 ml Acrylamide/Bis (38%/2%), 250 μl 25% APS, 60 μl TEMED

Tryptophan: 1% solution

Fluorescence Buffer: 50 mM Tris pH 8.0, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT

Annealing Buffer: 50 mM Tris pH 8.4, 80 mM KCl, 7 mM MgCl₂, 5 mM DTT
Supplies  (from Fisher unless otherwise noted)

Glassware includes: beakers, flasks, graduated cylinders, bottles, test tubes (Becton Dickinson), pasteur pipettes (Scientific Products)

Sterile plasticware: 15ml conical tubes, 50ml conical tubes, cryovials, petri dishes
Nonsterile plasticware: 1,000μl, 100μl, and 10μl pipet tips; 250ml, 50ml, and 15ml centrifuge bottles and tubes (Nalgene); 1.5ml and 0.5ml microcentrifuge tubes; disposable pipets; 1 liter culture flasks;

Stir bars
Culture loop and glass culture spreader
3ml, 10ml, and 60ml syringes (Becton Dickinson)
18 and 20 guage needles (Becton Dickinson)

Eppendorf adjustable pipets: 100-1,000μl, 10-100μl, and 0.5-10μl

Quartz cuvettes for DNA measurements
Quartz cuvettes for fluorimetry

Plastic cuvettes for cell culture and protein measurements

Tracer Blood Glucose Test Strips (Boehringer Mannheim)

pH Indicator Paper, Type CF (Whatman)

Dialysis tubing - 30,000 MW

Tissue culture filter, 250ml (Nalgene)
Sterile Acrodisc, 0.2μM (Gelman Sciences)
YM30 Diaflo ultrafilter membranes

Centricon-30 microconcentrators (Amicon)

Columns: 500ml, 50ml, 15ml (pharmacia)

Glass beads (Sigma)

Razor blades (VWR Graphics)

EDF film, Kodak (Promega)

Developing trays
Silver Sequence staining trays
Nitrogen gas (AIRCO)
Helium, high purity (AIRCO)

**Equipment**

Autoclave - Market Forge Sterilmatic
Bead beater - Biospec Products
Centrifuges: Eppendorf Microcentrifuge 5415C kept at 4°C
    Savant Speedfuge HSC10AC kept at room temperature
    Beckman Model J 2-21, rotors JA-14 and JA-20
    Beckman L-70 Ultracentrifuge, rotor VTi 65
Computer Software: CA Cricket Graph III - Computer Associates
    MacVector - IBI, Kodak
DNA sequencing apparatuses, 21cm x 40cm and 21cm x 50cm - BioRad
Dry bath, adjustable temp - Fisher
Econo Pump - BioRad
Electrophoresis Systems, FB 452 and FB 600 - Fisher Biotech
Fluorescence Spectrophotometer, MPF-44A - Perkin-Elmer
Fraction collector - BioRad
High Pressure Liquid Chromatograph: BioLC - Dionex
Integrator, 4400 - Dionex
Incubator - Fisher
Microwave - Amana
Mini Horizontal Electrophoresis Apparatus - Sratagene
Scintillation counter, model LS1701 - Beckman
SDS-PAGE apparatus, "Mighty Small" - Hoefer Scientific Instruments
Shaker, Orbit Environ - Lab-Line Instruments, Inc.
Spectrophotometer, Spectronic 1001 - Bausch & Lomb
Speedvac Concentrator SVC 100H and Refrigerated Condensation Trap - Savant
Stirred Ultrafiltration Cell - Amicon
Superose 12 FPLC column - Pharmacia
Thermocycler, Temp-Tronic - Barnstead/Thermolyne Corp.
Tube Sealer - Beckman
TSK G3000 SW HPLC gel filtration column - Tosohaas
UV light box: Fotodyne Incorp., Hoefer Scientific Instruments
Vortex - Fisher
Water bath - Fisher
METHODS

Yeast Plasmid Expression Vector, pAB24/RT5, and Original Yeast Strain, AB110

The RT gene that was used to produce wild type enzyme originally comes from a clone of the viral DNA prepared from a San Francisco isolate of HIV called ARV-2 (35, 46). The plasmid containing the RT gene, pAB24/RT5 (see Figure 4), was prepared by Bathurst (20) by modification of pAB24 /RT4, a plasmid constructed in 1987 by Philip Barr and coworkers (26). A 78 base pair synthetic oligonucleotide was ligated onto the end of the RT4 gene extending the coding region to Leu715. This made the gene code for the full length 66kD monomeric polypeptide (RT5) from Pro156 to Leu715, amino acid positions on the HIV pol open reading frame (20, 26). The RT expression cassette is flanked by BamH-1 and Sal-1 restriction enzymes so the cassette can be removed and manipulated using recombinant DNA technology.

pAB24/RT5 is an autonomously replicating plasmid which is designed to constitutively express the RT gene in yeast (20, 26). Saccharomyces cerevisiae strain AB110 (Mat α leu 2 trp 1 ura 3-52 pr B1-1122 pep 4-3 pr C1-407 [circ]) was used to express the gene for the wild type RT, RT5 (20). Expression is controlled by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter and terminator which flank the RT gene (26). When glucose levels in YPD media containing 1% glucose are used up by the yeast cells, the promoter is activated and expression of the RT gene begins. Yeast cells containing the plasmid are initially selected for by growing them in leucine minus rich media, which contains 8% glucose for 24 hours at 30°C. The plasmid contains a gene for leucine so only cells containing plasmids with this gene survive.
Figure 4. Diagram showing linear and circular plasmid maps of the yeast expression vector, pAB24. This plasmid is used for replication and expression of wild type RT5, mutant L289R, and mutant L289P. The RT gene is carried in a Sal-I/BamH-I cassette.
Once yeast cells containing pAB24/RT5 have been selected by growing in leucine minus media they are transferred to 1% glucose YPD media and grown for 30 hours at 30°C to induce expression.

New Yeast Strain, XS95-6C

For cloning the mutated enzyme a new yeast strain was used called XS95-6C (Matα rad52-1 his3-112 leu2-3 leu2-112 ura3-52 trp1-289a [cir0]). The main difference between these yeast cells and the strain used previously is that the new strain is not protease deficient so the new strain may produce a protease that will break down the RT as it is expressed. The methods used for determining efficiency of protein expression will be described later. First, an experiment was done to determine the growth curve for the XS95-6C yeast strain. Briefly, an overnight culture from 1 colony is grown in YPD medium. The cells are grown until the optical density at 600nm (OD600) is approximately 1.0. Three flasks containing 250ml YPD each are inoculated with the overnight culture, one with 500μl, one with 1ml, and one with 2ml. The cultures are incubated on a shaker at 37°C and a 1ml sample is removed from each culture approximately every 4 hours and the OD600 recorded.

Isolation of pAB24/RT5 from Yeast

Plasmid carrying the gene for wild type RT (RT5) must be isolated from the yeast strain AB110 so it can be used as template for PCR mutagenesis. This procedure is based on a method from Current Protocols in Molecular Biology (48). First grow an overnight culture by inoculating 3 ml of leucine-minus, rich media with 1ml stock culture. Incubate 24 hrs at 30°C on shaker. Put 1.5 ml of yeast culture into each of 2 microcentrifuge tubes. Pellet cells by centrifugation for 5 seconds at top speed, room temperature. Discard supernatant and resuspend cells in 200μl of breaking buffer. Add 200μl glass beads and 200μl phenol/chloroform/isoamyl alcohol (25:24:1). Vortex
highest speed for 2 minutes. Centrifuge 5 minutes, room temperature. Transfer the top layer to a fresh tube and ethanol precipitate the DNA.

**Ethanol Precipitation of DNA**

Ethanol precipitate the aqueous DNA solution with 2 parts 100% EtOH to 1 part aqueous sample plus 1/10 of the DNA sample volume of 3M sodium acetate solution pH 5.2. Store at least 2 hours at -20°C. Centrifuge 20 minutes at 4°C, top speed. Wash pellet with 70% EtOH and dry in Speed Vac. Resuspend plasmid DNA pellet in TE buffer.

**Prepare competent *E. coli***

Inoculate 100 ml of LB broth in a 500 ml flask with 1 ml of an overnight culture of HB101 cells. Grow cells with vigorous shaking at 37°C to a density of approximately 5x10^7 cells/ml (0.5 OD_{550}), usually about 2 to 4 hours. Use 3 mls for each transformation. Chill on ice for 10 minutes. Spin at 4,000g for 5 min at 4°C. Discard supernatant. Resuspend cells in half the original culture volume (if starting with 3 mls use 1.5 mls) of ice-cold, sterile solution of 50 mM CaCl₂ and 10mM Tris-Cl, pH 8.0. Place in ice bath for 15 minutes and spin 5 minutes at 4°C. Discard supernatant. Resuspend cells in 1/15 of the original volume (if started with 3 mls use 200 μls) of ice-cold, sterile solution of 50 mM CaCl₂ and 10mM Tris-Cl, pH 8.0. Dispense 200 μl aliquots into prechilled tubes. Store cells at 4°C for 12-24 hours before transformation or at -70°C indefinitely.

**Transformation of HB101 *E. coli* with pAB24/RT5 from Yeast**

The transformation of *E. coli* with plasmid is based on a procedure from the Maniatis laboratory manual (49). Add up to 40ng of DNA (dissolved in up to 100μls of TE buffer) to each tube of competent cells. Mix and incubate on ice 30 minutes.
Incubate at 42°C for 2 minutes then put back on ice. Add 1 ml LB broth (without ampicillin) and incubate 37°C for 1 hour without shaking. Set up dilutions and place 100μl of each dilution (plus 1 undiluted) on LB + ampicillin plates and spread evenly over entire plate.

Set up control plate using untransformed cells with no DNA added. Incubate 12-18 hours at 37°C, no longer, then store at 4°C. Make stock cultures of any colonies that grow and do plasmid miniprep on one of the stocks to do restriction mapping of the plasmid to confirm that pAB24/RT5 is present in the HB101 cells.

**Stock Culture Preparation**

Place 1 colony of transformed *E. coli* in 5 ml LB + ampicillin media. Incubate 37°C overnight on shaker. Add 5 ml 30% sterile glycerol for a final concentration of 15% glycerol. Place 250μl aliquots into cryovials and store at -70°C.

**Miniprep of Transformed *E. coli*, Alkaline Lysis Method**

The following procedure is based on the method described in the Maniatis lab manual (50). Take one of the above stock cultures and place in 5ml LB media with ampicillin. Incubate 37°C overnight on shaker. Put 1.5ml of the overnight culture in a microfuge tube and spin 1 minute to pellet cells. Store rest of culture at 4°C for up to 1 week. Discard supernatant. Resuspend pellet in 100μl of ice cold Solution I. Incubate 5 minutes, room temp, uncapped. Add 200μl fresh Solution II and invert rapidly 2-3 times (don't vortex). Incubate on ice 5 minutes. Add 150μl of ice cold Solution III and vortex gently while inverted. Incubate on ice 5 minutes. Centrifuge 5 minutes at 4°C. Transfer supernatant to a fresh tube. To purify the DNA first add an equal volume of Tris-buffered phenol pH 8.0. Mix by vortexing 5 minutes and then centrifuge at highest speed for 10 minutes at room temperature. Transfer the top aqueous layer to a fresh tube and add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Mix and spin as
above and transfer the top layer to a fresh tube. Add an equal volume of chloroform / isoamyl alcohol (24:1) and mix, centrifuge, and transfer top layer to fresh tube as above. EtOH precipitate the aqueous DNA sample as described above. Resuspend dry pellet in TE buffer which contains DNase-free pancreatic RNase (20μg/ml).

Quantitate DNA

Zero spectrophotometer with 1 ml of water or TE buffer, whichever the sample is in, at 260nm in glass cuvette. Record absorbance of the blank at 280nm. Add 1 μl of DNA sample and record absorbance at 260nm and at 280nm. Determine purity as follows: divided the absorbance at 260nm by the absorbance at 280nm. Determine the concentration as follows: absorbance at 260nm multiplied by 50μg/1 OD for double-stranded DNA, or 40μg for single-stranded DNA and RNA equals the concentration in μg/μl (51).

Restriction Enzyme Analysis of Plasmid

In order to confirm that I have obtained the correct plasmid from the transformed E. coli, the DNA is digested with restriction enzymes which will cut the DNA in specific places. The sample is then run on a 1% agarose gel and stained with ethidium bromide. The result will be a specific pattern of bands of different sizes. For digestions use 1-5 U of restriction enzyme per μg of DNA. BamH-1 is 16U/μl, Sal-1 is 8U/μl, and Hind-3 is 10U/μl.

1. BamH-1 + Sal-1 Digest

Combine up to 10μg Plasmid DNA in 7μl sterileTE or H2O, plus 1.0 μl 10X Buffer D, 1.0 μl BamH-1, and 1.0 μl Sal-1, in a 1.5ml microcentrifuge tube. Incubate 2 to 3 hours 37°C. Add 1.0 μl 10x DNA sample buffer and run on 1% agarose gel immediately or store at -20°C. Digest results in 2 fragments: 1) 2,096 bp and 2) 13,965 bp.
2. Hind-3 Digest

Combine up to 10µg Plasmid DNA in 7µl sterileTE or H$_2$O, plus 1.0µl 10X Buffer B, 1.0µl Hind-3, and 1.0 µl H$_2$O, in a 1.5ml microcentrifuge tube. Incubate 2-3 hours 37°C. Add 1.0µl 10x DNA sample buffer. Run on 1% agarose gel immediately or store at -20°C. Digest should result in 6 fragments: 1) 1,166 bp, 2) 2,107 bp, 3) 4,285 bp, 4) 3,430 bp, 5) 1,314 bp, 6) 2,659 bp.

1% Agarose Gel Electrophoresis

1.0g low gelling temperature agarose is dissolved in 100ml TAE buffer by heating in a microwave. The solution is allowed to cool slightly and poured into a Stratagene gel casting apparatus. Once the gel solution has hardened the gel is covered with TAE buffer, the dams and comb removed, and DNA samples which have been mixed with DNA sample loading buffer/tracking dye are loaded into wells. Lambda DNA BstE II Digest is used as a size marker. The gel is run at 100 volts or less. The gel is stained in ethidium bromide solution (0.5µg/ml final concentration) for 10 minutes and then rinsed in dH$_2$O for 30 minutes. Using a UV light box to illuminate the stain, a photograph can be taken to record the image and, if desired, bands can be cut out of the gel and purified for later manipulation.

PCR Mutagenesis

Site-directed mutagenesis, also called site-specific mutagenesis or oligonucleotide directed mutagenesis will be used to correlate specific changes in the DNA sequence with effects on function of RT. There are many different methods of site-directed mutagenesis which can be used to change a single amino acid of a cloned protein (52). One of the newest mutagenesis methods uses the polymerase chain reaction (PCR) which is very quick, simple, and efficient compared to traditional methods. The first use of PCR to amplify DNA was in 1985 (53). It was immediately realized that PCR could be used to
engineer DNA in many different ways (54, 55, 56). For example, PCR could be used to introduce restriction sites (54), to make base substitutions (57, 58), to make insertions or deletions (59), or to assemble overlapping oligonucleotides into long synthetic DNA fragments (60). Since our lab is equipped with a thermocycler which is necessary for PCR procedures, this method of mutagenesis is the most practical for our lab to use. A drawback of PCR mutagenesis has been that it used Taq polymerase which does not possess 3' to 5' exonuclease activity and, consequently, cannot correct misincorporation. The error rate of Taq polymerase is estimated to be about one error every 900 bases (61, 62). Recently, a new PCR polymerase enzyme has been put on the market by Stratagene called Pfu polymerase which does have proofreading ability and, according to the manufacturer, shows a twelve fold increase in fidelity over Taq. I have designed a procedure for creating a base pair substitution at amino acid position L289 of HIV-1 RT using PCR mutagenesis. The outline for this procedure is shown in Figure 5.

PCR mutagenesis requires four synthetic oligonucleotide primers designed to bind specific strands of template DNA at strategic sites. Primer binding sites depend on where unique restriction sites are in the RT gene and where the desired sequence change is located. As can be seen in Figure 5 the outside primers, P1 and P6, are designed to be adjacent to AlwN-1 and Sal-1 restriction sites of the RT gene so that the PCR mutagenesis product can be cut and inserted into the plasmid vector. The inside overlapping mutagenic primers, P2 and P3 for mutant #1 or P4 and P5 for mutant #2, are complimentary to each other at the 5' end but mismatch the template at the site of the desired base change. The design of the oligonucleotide primers is critical to successful PCR mutagenesis and must be chosen carefully. They should be at least 20 base pairs in length or have a GC content of 50% or greater, and the overlapping region between the two inside primers should be at least 20 base pairs. The 6 primers used for creating the two mutants are shown in Figure 6. The RT DNA and amino acid sequences are shown in Figure 7 along with primer binding sites and restriction enzyme sites.
Figure 5. Diagram showing the procedure for using the polymerase chain reaction (PCR) to create a site-specific mutation in the RT gene.
1st PCR: two separate reactions

Isolate and purify desired PCR fragments

2nd PCR: Mix these two fragments together and add outside primers p1 and p6

Cut PCR mutated fragment with AlwN-1 and Sal-1
**Figure 6.** Primers used for making two RT mutants using PCR mutagenesis. Primers 1 and 6 are the outside primers used for both mutations. Primers 2 and 3 are the inside mutagenic primers for creating the L289P mutation. Primers 4 and 5 are the inside mutagenic primers for creating the L289R mutation.
<table>
<thead>
<tr>
<th>Primer #</th>
<th>Sequence</th>
<th>%GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5' GAGGAACTGAGACAGC 3'</td>
<td>(16)</td>
</tr>
<tr>
<td>2</td>
<td>5' GGTATTACTTCTGTGGTGCTTTGG 3'</td>
<td>(25)</td>
</tr>
<tr>
<td>3</td>
<td>5' GCACCAACAGAAAGTAATACC 3'</td>
<td>(20)</td>
</tr>
<tr>
<td>4</td>
<td>5' GGTATTACTTCTGTGGTGCTTTGG 3'</td>
<td>(25)</td>
</tr>
<tr>
<td>5</td>
<td>5' GCACGAACAGAAAGTAATACC 3'</td>
<td>(20)</td>
</tr>
<tr>
<td>6</td>
<td>5' GTCGACTATAGTACTTTCC 3'</td>
<td>(20)</td>
</tr>
</tbody>
</table>

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Figure 7. DNA and amino acid sequence of the RT gene. The sites for PCR primer binding are shown as well as the restriction enzyme sites used to place the mutated fragment into the plasmid expression vector.
The exact concentrations of ingredients for the PCR reaction mixtures was determined through experimentation. Generally, the 100μl reaction mixture contained: 2.5 U Pfu DNA polymerase, 1μM of each primer, 0.1 to 250ng template DNA, 300μM of each dNTP, and 10μl of 10x pfu buffer. DNA template samples that contain EDTA, such as TE buffer, should use a 10x pfu buffer that contains 20mM MgCl₂. The cycle conditions depend on the DNA template, the DNA primer, and the desired product.

Amplification of fragments A and B

Turn on thermocycler 1/2 hour before using, and program it as follows: dwell at 95°C for 10 minutes, denature at 95°C for 5 minutes, anneal at 55°C for 5 minutes, extend at 72°C for 5 minutes, dwell at 4°C. Set up the following reaction mixes:

For fragment A (265 bp):

- 24μl sterile, ddH₂O
- 10μl 10X pfu buffer #1
- 60μl dNTP mix
- 130ng plasmid, pAB24/RT5
- 2μl of 50μM Primer #1
- 2μl of 50μM Primer #2

For fragments B (829 bp):

- 24μl sterile, ddH₂O
- 10μl 10X pfu buffer #1
- 60μl dNTP mix
- 130ng plasmid, pAB24/RT5
- 2μl of 50μM Primer #3
- 2μl of 50μM Primer #6

Set up a negative control as above except without DNA template. Put 1 AmpliWax PCR Gem100 pellet into each tube. Begin program described above on thermocycler. After an initial 10 minute dwell, add 1μl pfu polymerase to each tube and resume program for 30 cycles. Once the program has finished add 10μl of 10x DNA sample buffer to each tube and store at -20°C.

Isolate and Purify Fragments A and B

Run PCR reaction mixture along with DNA size markers on a 1% agarose gel. Using a UV light box to illuminate the stain, cut out desired bands with a razor blade and

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place in 1.5ml microcentrifuge tubes. Each tube should contain about 100 μl of agarose containing DNA. Add 400μl of TE buffer to each tube and heat to 65°C to melt agarose. Add 500μl of phenol and vortex at low speed for 5 minutes. Centrifuge at top speed for 10 minutes to separate layers. Collect top aqueous layer and re-extract with phenol. Add 500μl of TE buffer to bottom phenol layer and re-extract. Combine aqueous layers and ethanol precipitate as described above. Resuspend pellet in TE buffer and quantitate DNA as described above.

**Synthesis of Mutant Fragment C**

First, create a linking program on the thermocycler made by linking two different programs together. The first program is designed to enhance annealing of the two fragments A and B which have 20 complimentary base pairs at their 5-prime end (+ strand). When the two fragments are combined in the PCR reaction mixture with primers and then denatured, there are six possible recombinations which are shown in Figure 8

Extension of annealing product #6 will result in the new 1,084 bp fragment C. The first part of the two part program is as follows: Dwell at 95°C for 10 minutes, denature at 95°C for 1 minute, anneal at 45°C for 4 min, and extend at 72°C for 2.5 minutes, for 4 cycles. Link this program to the following program: denature at 95°C for 1 minute, anneal at 50°C for 2 minutes, and extend at 72°C for 2.5 minutes, for 25 cycles. Dwell at 4°C until morning. Fragment C is isolated from the PCR mixture as described above for fragments A and B and digested with AlwN-1 and Sal-1 as described below.

**AlwN-1 + Sal-1 Digest**

To 8μl of fragment C DNA in TE buffer add 1μl of AlwN-1 enzyme and 1μl of Promega Buffer E in a 1.5ml microcentrifuge tube. Incubate 2 hours then add 2.5μl of H₂O, 1.5μl of Promega Buffer D, and 1μl of Sal-1 enzyme and incubate for 2 more hours.
Figure 8. Diagram showing the six possible fragments produced during the formation of fragment C in PCR mutagenesis. Extension of #6 will result in a double stranded DNA fragment containing the desired mutation.
Product #1 = PCR Fragment A:
5' _______________ 3'
3' -------------------- 5'

Product #2 = PCR Fragment B:
5' ______________________ 3'
3' -------------------------- 5'

Product #3:
5' P1 3'
3'----------------------5'

Product #4:
5' ______________________ 3'
3' ----------- 5'
P6

Product #5:
5' ______________________ 3'
3' ---------------------- 5'

Product #6:
5' _______________ 3'
3' ________________________ 5'
Add 2μl of DNA sample buffer and run on a 1% agarose gel. Isolate and purify the fragment from the gel as described above.

**Method for Putting Mutant Fragment C into Expression Vector**

The procedure for placing the mutated fragment C back into the yeast expression vector, pAB24, is summarized in Figure 9. The first step of digesting plasmid pAB24/RT5 with BamH1 and Sal-1 restriction enzymes was described above. The digest is run on a 1% agarose gel and the large and small BamH-1/Sal-1 fragments isolated and purified from the gel. The large fragment is stored at -20°C for later use. The small fragment is digested with AlwN-1 and Pvu-2 as described below. The Pvu-2 digest serves to divide the AlwN-1/Sal-1 fragment into smaller pieces so that it will not be confused with the BamH-1/AlwN-1 fragment which is the same size. This makes isolation of the BamH-1/AlwN-1 fragment in 1% agarose gel easier (see Figure 9).

Combine 10μl of the small BamH-1/Sal-1 fragment (1μg/μl), 1.5μl H2O, 1.5μl 10 x Promega Buffer C, 1μl AlwN-1, and 1μl Pvu-2 and incubate at 37°C for 2 hours. Run this digest mixture on a 1% agarose gel to separate the fragments by size as follows: BamH-1/Sal-1 (2,090), BamH-1/AlwN-1 (1,025bp), AlwN-1/Pvu-2 (135bp), Pvu-2/Sal-1 (830bp). Isolate and purify the BamH-1/AlwN-1 fragment as described above and resuspend in TE buffer. Ligate the BamH-1/AlwN-1 fragment with the PCR mutated AlwN-1/Sal-1 fragment as follows: Combine 2.5μl H2O, 5μl (2pmol) of the AlwN-1/Sal-1 fragment (0.3μg/μl), 5μl (14.8pmol) of the BamH-1/AlwN-1 fragment (2.0μg/μl), 1.5μl ligase buffer, and 1.0μl ligase and incubate at 4°C for 48 hours. Add 1μl (1pmol) of the BamH-1/Sal-1 fragment (11μg/μl) and incubate another 48 hours. Set up several transformation reactions using different dilutions of the ligation mixture and 200μl of competent HB101 *E. coli* cells per 1.5ml tube (see procedure described above). Transformation mixtures are plated on LB+ ampicillin plates and incubated at 37°C. *E. coli* that grow on the selective plates contain the mutated RT plasmid vector.
Figure 9. Diagram showing how the PCR mutated fragment was inserted into the yeast expression vector, pAB24. The original plasmid vector carrying the gene for wild type RT, pAB24/RT5, was digested with restriction enzymes in order to remove the fragment corresponding to the PCR mutated fragment which has been digested with AlwN-1 and Sal-1. Ligating the desired digestion products together results in the formation of a new plasmid containing the site-specific mutation, pAB24/RT5M.
Plasmid: pAB24/RT5

RT gene

16,055

Digest with BamH-1 and Sal-1

PCR mutated fragment digested with AlwN-1 and Sal-1

BamH-1

Sal-1

1,065

Isolate, digest with AlwN-1 and Pvu-2

BamH-1

AlwN-1

Pvu-2

Sal-1

1,025

225

840

Isolate

Ligate to mutated fragment

Plasmid: pAB24/RT5M

Transform HB101 cells

BamH-1

Sal-1

2,090

1,025

225

840
MaxiPrep of mutant pAB24/RT5 from Transformed E.coli

To obtain large amounts of plasmid for sequencing and transformation into yeast, a large scale preparation using cesium chloride gradient ultracentrifugation was used. Grow up a liter of *E. coli* cells containing the mutated plasmid in LB + ampicillin medium. Harvest the cells by centrifugation at 4,000 x g (6,000 rpm) in 250ml centrifuge bottles in JA-14 rotor at 4°C for 10 minutes. Discard supernatant and resuspend each pellet (from 250mls of culture) in 5ml ice cold Solution I and incubate uncapped 5 minutes at room temperature. Add 10ml fresh Solution II close top, invert rapidly 2-3 times, and store on ice 5 minutes. Add 7.5ml ice cold Solution III close top and vortex inverted 10 seconds. Store on ice 5 minutes. Centrifuge 15,000rpm in JA-20 rotor 15 minutes at 4°C and transfer supernatant to a clean tube. Spin again at 15,000rpm for 10 minutes and transfer supernatant to a clean tube. Add 10ml phenol, mix, centrifuge 3,000rpm for 10 minutes and transfer aqueous (top) layer to a clean tube. Add 9.45ml isopropanol, mix, let stand 5 minutes at room temperature then spin 12,000 rpm for 10 minutes. Discard supernatant and wash pellet with 70% EtOH. Dry pellet and resuspend in TE buffer. Run sample on 1% agarose gel to see if plasmid is present. Set up the cesium chloride gradient using two DNA samples or one sample and a buffer blank to use as a balance. Put DNA sample in a tube and add H₂O so final volume is 4.1ml. In a 50ml tube add 4.2g cesium chloride and the DNA solution. Mix, add 150μl of stock ethidium bromide solution and mix again avoiding bubbles. Take the plunger out of a 3ml syringe, put on an 18 gauge needle, and place the needle of the syringe into the opening of an ultracentrifuge tube. Pour the DNA-cesium chloride mixture into the syringe so it fills up the ultracentrifuge tube without any bubbles. Balance tubes to within 0.05g. Seal tubes and place in ultracentrifuge rotor according to manufacturers instructions. Samples are spun overnight at 55,000 rpm and 22°C. DNA is visualized using a UV light and removed using a needle and syringe after first making an air hole in the top of the centrifuge tube with the needle. The DNA is separated from ethidium.
bromide by extracting repeatedly with a 5M NaCl saturated N-butanol solution. Once the bottom aqueous layer containing the DNA is no longer pink, the DNA is precipitated by adding 2 volumes of H$_2$O, 6 volumes of 100% EtOH, and 1/20 volume 3M sodium acetate, pH 5.2. Run the DNA sample on a 1% agarose gel to determine if RNA is present. If RNA is present then the sample must be treated with RNase and the phenol extraction and ethanol precipitation procedures repeated to remove the RNase. The RNA can also be removed using GeneClean according to the manufacturer's instructions.

**DNA Sequencing**

There are several methods for direct sequencing of PCR products which use different ways of generating single-stranded DNA for sequencing (64, 65, 66). After first trying USB's Sequenase kit and Promega's Fmol kit it was found that Promega's Silver Sequence method, which uses asymmetric PCR amplification of the DNA template, was the most successful. The Silver Sequence method detects bands in a DNA sequencing gel using a sensitive silver staining procedure and does not require the use of any radioactive materials. The stained bands can be observed with the naked eye but reading and recording of the sequence is aided by placing the stained gel on top of a fluorescent light box. The overall sequencing reaction is based on Sanger's enzymatic dideoxy sequencing method (67, 68) which requires single-stranded template DNA. A variation of Sanger's enzymatic sequencing method uses thermocycling and thermostable taq DNA polymerase which allows the use of double-stranded DNA as template. Thermocycling also improves specificity of primer hybridization and decreases formation of secondary structures of the DNA template (69).

The first step of any sequencing method is to obtain very clean DNA which was done using the maxiprep method described above. For Silver sequencing 2pmol or 22μg of plasmid DNA was used. By following the manufacturer's instructions and using high purity H$_2$O and sodium carbonate, and by not rinsing too long after the staining
procedure, the method was usually successful. Briefly, for each sequencing reaction set up four 0.5ml tubes labeled A,C,G, and T and pipette 2µl of each ddNTP mix into the appropriately labeled tube. Add one drop of oil to each tube then place tubes on ice. In a separate tube combine 6.5µl sterile H2O, 5.0µl 5x sequencing buffer, 4.0µl DNA (5.25µg/µl), and 0.5µl of 50µM primer. The same primers that were used for PCR mutagenesis were used for DNA sequencing. 1 µl of Taq DNA polymerase was added to the sequencing reaction mix which brings the total volume to 16 µl. Pipet 4µl of this mixture into each of the 4 ddNTP tubes. Spin the tubes briefly and place in the thermocycler preheated to 95°C which has been programmed as follows:

Dwell at 95°C for 2 minutes, then
Step 1: 95°C for 0 seconds
Step 2: 95°C for 30 seconds (denaturation)
Step 3: 42°C for 0 seconds
Step 4: 42°C for 30 seconds (annealing)
Step 5: 70°C for 0 seconds
Step 6: 70°C for 30 seconds (extension)
for 60 cycles.

Samples can be kept in thermocycler dwelling at 4°C for overnight if desired. Add 3µl of stop solution to each tube and store on ice or at -20°C. Heat samples at 70°C for 2 minutes before loading onto a 6% polyacrylamide gel containing 8M urea. For silver staining the sequencing gel the plates must be specially prepared. The short plate is made sticky using a binding solution prepared with Bind Silane which comes with the kit. The long plate is made slippery with either Sigmacote or Rainex, bought separately and used according to manufacturer’s instructions. Care is taken to make sure plates are extremely clean before preparing and not to cross-contaminate plates by changing gloves as the kit suggests. The gel rig is assembled and the gel poured according to manufacture’s directions, except the bottom is sealed differently. A spacer is cut to fit across the bottom in between the two side spacers and, unlike the side spacers, is coated with silicon grease to insure that no leakage occurs through the bottom while pouring the gel.
60mls of a 6% acrylamide gel solution is made as described in the Materials section. 10mls of this solution was set aside and to the remaining 50mls add 170μl of a 25% ammonium persulfate (APS), and 45μl of TEMED to catalyze polymerization. After the main part of the gel has polymerized the bottom spacer is removed. 42μl of 25% APS and 10μl of TEMED is added to the 10mls of acrylamide solution previously set aside and the solution is poured in to fill the space at the bottom of the gel. Originally, a square toothed 16 well comb was used, but this was replaced with a 24 well sharks toothed comb which results in lanes that are much closer together and thus easier to read. Two different size apparatuses were used for sequencing, spacer thickness for both was 4mm. The first apparatus was 21cm x 40cm and was run at 1800volts, 55watts, and 35mAmps. The second apparatus was 21cm x 50cm and was run at 2000volts, 60watts, and 35mAmps. The temperature was closely observed and settings adjusted when necessary to maintain a running temperature of 50°C. Gels were pre-run until the temperature reached at least 45°C before loading samples. The length of time for each run depended on which region of the sequence was being read. A maximum of 200 bases could be read on the shorter apparatus after running the gel 4 hours. The longer gel was run 4 to 8 hours and up to 350 bases could be read. Gels were stained according to Promega's Silver Sequence instruction manual and the image recorded as follows. In a dark room place the gel attached to the glass plate on top of a fluorescent light box turned off. Place a piece of Kodak EDF film on top of gel, turn light box on for 1-2 seconds and turn off. Place film in developer solution for 2 minutes, rinse in water for 1 minute, place in fixative for 3-5 minutes, then rinse in water and hang to dry.

**Yeast Transformation**

The spheroplast method of yeast transformation (70) was used to place mutated pAB24/RT5 into yeast strain XS95 for expression and purification of mutant enzymes, L289R and L289P. This method uses the enzyme β-glucuronidase to remove the cell wall.
from the yeast cells, making them spheroplasts, so that DNA can pass through the cell membrane. The procedure can be divided into two parts, 1) the formation of spheroplasts, and 2) the transformation of spheroplasts. First sterilize the following glass/plastic ware: (1) 10-15ml test tube for growing the overnight culture, (4) 250ml flasks, (4) 50ml round bottom centrifuge tubes, (1) 50ml flask, (6) 10ml glass test tubes. Prepare the following solutions as described in the Materials section: 1M sorbitol, CaCl$_2$ solution, sorbitol/CaCl$_2$ solution, filter sterilized PEG/CaCl$_2$ solution, and β-Glucuronidase (90,000 U/ml). Prepare at least 200mls YPD liquid medium and 2 YPD plates. Prepare 500mls leucine minus selective regeneration agar. Prepare 500mls leucine minus medium for selective plates. To prepare spheroplasts, first grow an overnight culture by inoculating 2mls of YPD with 1 colony of the XS95 yeast and grow overnight at 30°C on a shaker. The next day use different amounts of this culture to inoculate 4 different 250ml flasks containing 50mls of YPD as follows: 1) 100μl, 2) 250μl, 3) 500μl, and 4) 1ml of overnight culture. Grow until OD$_{600}$ of one of the cultures reaches 0.5, about 18 hours. The next day melt the selective regeneration agar and aliquot 10mls into each of 6 sterile test tubes and place in 55°C water bath. Take the culture which has an OD$_{600}$ that is closest to 0.5 and pellet in 50ml sterile centrifuge tube by spinning at 1100 x g (3500 rpm) 5minutes, room temperature in JA-20 rotor. Discard supernatant and resuspend pellet in 10ml 1M sorbitol. Repeat this step. Pellet cells again and resuspend in 5mls of 1M sorbitol. Take 1μl of this cell suspension and make a 10$^{-5}$ dilution in sterile H$_2$O. Plate 100μl of this dilution on a YPD plate and incubate 2 days at 30°C. This plate will indicate cell viability before β-Glucuronidase treatment. Put yeast cell/sorbitol suspension in a 50ml sterile flask and add 5μl of β-mercaptoethanol and 150μl of β-Glucuronidase (13,500 U). Incubate 60 minutes at 30°C with very gentle shaking. Take 1μl of this cell suspension and make a 10$^{-3}$ dilution in sterile H$_2$O and plate 100μl on a YPD plate and incubate 2 days at 30°C. If spheroplasts are present they will lyse in H$_2$O and will not grow on the plate. Calculate % of cells that did not form spheroplasts as.
follows: number of colonies after β-Glucuronidase treatment divided by number of colonies before β-Glucuronidase treatment. Transfer the β-Glucuronidase treated cell mixture to a sterile 50ml round bottom centrifuge tube and pellet by spinning 4 minutes at 400 x g. Gently decant supernatant without dislodging pellet. Add 2ml of 1M sorbitol and resuspend by gently swirling. Add 8mls of 1M sorbitol and pellet at 400 x g. Decant supernatant, add 2mls of 1M sorbitol and resuspend by swirling. Add 8mls of 1M sorbitol and pellet by spinning 4 minutes at 400 x g. Decant supernatant, add 2mls of 1M sorbitol and resuspend by gently swirling. Add 7mls of 1M sorbitol and 1ml of the CaCl$_2$ solution. Mix gently by swirling and centrifuge 4 minutes at 400 x g. Decant supernatant and resuspend the pellet in 1ml of the sorbitol/CaCl$_2$ solution. Set up 6 sterile microcentrifuge tubes and place 150μl of yeast spheroplasts into each tube. Add varying amounts of DNA, up to 10μg in ≤ 20μl TE buffer as follows: tube#1) 0μg DNA, tube#2) 0.5μg DNA, tube#3) 1μg DNA, tube#4) 2μg DNA, tube#5) 5μg DNA, tube#6) 10μg DNA. Mix DNA with cells and incubate 10 minutes at room temperature. Add 1.5mls of PEG/CaCl$_2$ solution thoroughly resuspend and let sit 10 minutes at room temperature. Pellet cells, 4 minutes at 400 x g and decant off PEG/CaCl$_2$ solution. Gently resuspend pellet in 0.5ml sorbitol/CaCl$_2$ solution and pipet into 10mls of the melted regeneration agar which should be at 55°C. Vortex briefly and immediately pour onto the leucine minus selective plates and swirl to further mix. Incubate at 30°C until colonies appear. Make overnight cultures from single colonies picked from selective plates, and make glycerol stock cultures from the overnight cultures for further analysis.

**Yeast Expression Experiment**

The plasmid containing the mutated gene, pAB24/RT5M, is exactly the same as the plasmid containing the wild type gene, pAB24/RT5 except for a single base pair substitution in the subunit binding domain of the RT gene. Once the mutated gene was constructed, placed back into the plasmid expression vector, and transformed into the
new yeast strain, the best conditions for growing the new yeast strain and expression of the mutated gene were determined by experimentation. Briefly, 250 mls YPD medium in a 1 liter flask was inoculated with 25mls of the new yeast strain XS95 (transformed with pAB24/RT5M, grown in leucine minus medium) and incubated at 30°C on the shaker. 1.5ml aliquots of the culture were taken every hour for 24 hours and the following analyses done on each sample: the optical density read at 600nm wavelength, the glucose level measured, the pH measured, and the sample stored at -20°C to be analyzed later for RT activity and protein concentration. Mini-protein preps were carried out on the 26 samples using a glass bead disruption method (71) described below.

**Mini-multiple Protein Preps**

Each 1.5ml sample was spun 5 minutes at 4°C and the packed cell volume was determined to be approximately 10μl. Cells were resuspended in 30μl of H₂O, centrifuged, and the supernatant was removed. The cells were then resuspended in 30μl of disruption buffer plus 40μl of glass beads. The cell mixture was vortexed 1 minute at 4°C and stored on ice for 2 minutes. This was repeated 3 times. The cell mixture was centrifuged 5 minutes at 4°C and the supernatant collected. 200μl of disruption buffer was added, the mixture was spun again and the supernatant collected and combined with the first supernatant. Each sample was tested for RT activity and protein concentration as described below.

**Large Scale Protein Isolation and Purification**

Procedures for isolation and purification of the RT enzyme described below are the same for wild type and mutant RT.

**Harvest and Lyse cells**

Collect the cell pellets from several 1 liter yeast cultures by centrifuging the
culture at 10,000 rpm for 20 minutes at 4°C. If pellets have been frozen, place them in a large beaker and add enough RT lysis buffer to cover the cell pellets and wait until pellets thaw. Put mixture in the bead beater cylinder, add an equal volume of glass beads, and add buffer to the top so there is no air in the container. Beat mixture for 15 seconds, let cool for 1 minute, and repeat until total bead beating time on is 5 minutes. Pour solution into centrifuge bottles and spin at 10,000 rpm for 30 minutes at 4°C. Pour supernatant into large beaker. Add more lysis buffer to centrifuge bottles containing glass beads, swirl around to wash beads, and centrifuge again. Combine the two supernatants, record volume, and remove a 500μl aliquot of the crude extract to be analyzed later for activity and protein concentration.

**Protein Precipitation**

Put the beaker containing the cell extract inside another beaker packed with ice. Add 39.8g of ammonium sulfate for every 100ml of crude extract - adding it slowly while stirring the solution to precipitate out the protein. Centrifuge the solution at 10,000 rpm for 20 minutes at 4°C. Discard the supernatant and resuspend the pellet in 10 mls or less of RT buffer. Put the precipitated protein solution into sterile 30,000 MW cut off dialysis tubing. Dialyze overnight, changing buffer once, against 2 liters of 1x RT buffer to remove salt. After dialysis, dilute the protein solution 1:1 with RT buffer, remove a 500μl aliquot for later testing.

**Ion Exchange Column**

Whatman P11 cellulose phosphate is a cation exchanger with an ester-linked phosphate functional group which will bind or release the RT depending on the salt concentration of the running buffer. The phosphorylated cellulose was prepared by first washing with 0.5M NaOH and then 0.5M HCl and resuspending in 10x Tris buffer pH 7.5. A 500ml column is used for large preps but a smaller 50ml column was used for the
mutant purifications. The cellulose solution was packed into the columns and equilibrated with RT buffer at 4°C. After loading the sample, the column was washed with one bed volume of RT buffer. Save the "load" and "wash" eluants for testing. Prepare a salt gradient of 50 to 800mM KCl in RT buffer, 400mls each. RT5 elutes between 300 and 400mM KCl. Collect fractions using a BioRad fraction collector set for 25 minutes/tube while running the gradient. The BioRad Econo Pump is set at 20mls per hour. Assay the fractions for RT activity and protein concentration (see below) along with crude extract, "load" sample and "wash" sample to determine which fractions contain enzyme and what is the efficiency of purification. The results are plotted using Cricket Graph III on a double y axis graph showing protein concentration and absorbance versus fraction number. Peak fractions containing the most RT are pooled, concentrated, and loaded onto an Affi-Gel Blue column.

Comparison of Ion Exchange Column Purifications

To determine if the mutant purification results from the P11 column were significantly different from wild type purification, the above procedure was repeated three times for each mutant including enzyme activity analyses and protein concentration determinations. The results were averaged, graphed, and the salt concentrations that each mutant eluted at were determined and compared with results of wild type RT5 obtained previously.

Affi-Gel Blue Column

Affi-Gel Blue affinity gel is a crosslinked agarose bead with Cibacron blue F3GA dye covalently bound to it. The dye probably has an affinity for the nucleotide binding site of RT. The gel comes as a wet suspension which is stored and used at 4°C. The gel was packed into a 1.5cm x 10cm column and equilibrated with running buffer which is RT buffer without KCl. The column, which has a 15ml bed volume, can be loaded
with 1mg to 3mgs of protein per ml of Affi-Gel Blue so that a maximum of 45mg of protein could be loaded at a time. The protein solution was loaded and washed with no salt RT buffer until the optical density (O.D.) at 280nm of the eluant was consistent. Once protein has been loaded onto the column, RT is separated from other proteins by elution with a 0-600mM KCl in RT buffer gradient. Fractions were collected and assayed for RT activity and protein concentration.

**Superose 12 Column**

Further purification is obtained by loading the RT sample onto a Superose 12 FPLC gel filtration column at room temperature. Column settings are as follows: AUFS = 0.5, flow rate = 0.2ml/min, and high pressure limit = 430psi. Dionex integrator settings are as follows: peak threshold (PT) = 400,000, chart speed (CS) = 0.1, Attenuation (AT) = 1024. The integrator was programmed as follows:

<table>
<thead>
<tr>
<th>TT</th>
<th>TF</th>
<th>TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>&quot;T4&quot;</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
<td>&quot;T6&quot;</td>
<td>1</td>
</tr>
<tr>
<td>0.01</td>
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<td>1</td>
</tr>
<tr>
<td>90</td>
<td>&quot;T6&quot;</td>
<td>0</td>
</tr>
</tbody>
</table>

[TT = time in minutes, TF = function, TV = value: 1 means "on" and 0 means "off"]

A 25μl sample loop was used and samples containing 0.5 - 1.0μg/μl protein were injected at a time. Samples injected onto the column are monitored by UV absorbance at 280nm. All samples are either spun at 1400rpm for 5 minutes or filtered using microcentrifuge filtration devices to remove particles and bacteria before injecting them into the column. First, 25μl of BioRad Gel Filtration Standards are injected and a standard curve of log of molecular weight versus retention time is plotted to determine the expected retention time of the RT. Also, buffer is injected by itself to identify background peaks. Fractions are collected and assayed for enzyme activity. Samples containing RT are pooled, concentrated, and stored at -70°C.
TSK HPLC

For greater purification and analyses of protein based on molecular size, two TSK G3000 SW HPLC gel filtration columns were set up in series. Columns were equilibrated with TSK buffer and run at room temperature. The high pressure limit was set at 760psi, the maximum flow rate used was 0.3ml/minute, and AUFS was 0.5. As with the Superose 12 column, PT = 400,000, CS = 0.1, and AT = 1024. The integrator was programmed as follows: (for interpretation see previous page)

\[
\begin{align*}
TT(1) &= 0.01 \quad TF(1) = "T4" \quad TV(1) = 1 \\
TT(2) &= 0.01 \quad TF(2) = "T6" \quad TV(2) = 1 \\
TT(3) &= 0.01 \quad TF(3) = "PM" \quad TV(3) = 1 \\
TT(4) &= 0.1 \quad TF(4) = "T4" \quad TV(4) = 0 \\
TT(5) &= 120 \quad TF(5) = "ER" \quad TV(5) = 1 \\
TT(6) &= 120 \quad TF(6) = "T6" \quad TV(6) = 0
\end{align*}
\]

RT samples of 0.1 to 0.5µg/µl are loaded onto the column using a 25µl sample loop and were monitored by UV absorbance at 280nm. All samples are either spun at 1400rpm for 5 minutes or filtered using the microcentrifuge filtration devices to remove particles and bacteria before injecting them into the column. 25µl of BioRad Gel Filtration Standards were injected and a standard curve of log of molecular weight versus retention time was plotted to determine the expected retention time of the RT. Fractions are collected and assayed for enzyme activity. Samples containing RT are pooled, concentrated, and stored at -70°C.

**Protein Concentration Determination**

Protein concentration of fractions was determined by the Bradford Assay (72) using the Pierce Protein Assay kit. First, a standard curve is set up using dilutions of an albumin standard (2mg/ml) in distilled water in a total volume of 1ml. Usually, 0, 5, 10, 15, 20, and 30µg standard solutions were used. Add 1ml of Pierce Protein Reagent,
mix, and read absorbance at 595nm. Make a sample blank using the same volume of RT buffer as will be used to measure the sample, plus water to 1ml. Add 1ml of Pierce Protein Reagent, mix, and read absorbance at 595nm. The standard curve is plotted using Cricket Graph III and a linear equation is obtained and used to calculate the concentration of the sample.

**RT Polymerase Activity Assay**

To identify samples containing RT, samples were screened for their ability to incorporate $[^3H]$-dTTP using (rA)-(dT)$_{12-18}$ as a template. Reactions are started by adding 10µl of enzyme solution to 40µl reaction cocktail which contains final concentrations of 50mM Tris pH 8.5, 6mM MgCl$_2$, 80mM KCl, 8mM DTT, .05% Triton-X 100, 100µM dTTP, 2µg (rA)-(dT)$_{12-18}$, and 1µl (1µCi) $[^3H]$-dTTP. Samples are incubated at 37°C for 30 minutes. Reactions are terminated by adding 800mls of ice cold stop solution and pipetted onto GF/C filters. Filters are washed with 6mls of stop solution followed by 1ml of 95% EtOH. Filters are placed in scintillation vials and 6mls of scintillation fluid added. For each assay, count one vial that has 10µl of reaction cocktail plus 6mls of scintillation fluid. Vials are counted on a liquid scintillation counter and counts per minute (CPM) are converted to activity as follows:

$$\frac{\text{CPM of the sample}}{\text{CPM of 10µl of reaction cocktail}} = \text{activity (nmoles of }[^3H]\text{-dTTP incorporated) per 10µl of sample.}$$

Units of RT activity are expressed as nmoles of $[^3H]$-dTTP incorporated per ml.

**Concentrating Protein Sample**

The samples are concentrated using an Amicon Stirred Ultrafiltration Cell with an Amicon YM30 Diaflo ultrafilter membrane and nitrogen gas at 35 PSI. Fractions can be further concentrated using Amicon Centricon-30 microconcentrators spun in a Beckman model J2-21 centrifuge using a JA-20 rotor at 4.5rpm for 60 minutes or more.
TCA Precipitation

In order to analyze dilute samples using SDS-PAGE, an aliquot of the sample is concentrated by precipitation with 10% TCA. Take 450μl of the sample and add 50μl of 100% TCA. Spin the sample at highest speed for 5 minutes at 4°C. Wash the sample twice with 1ml of 95% EtOH and Speed Vac to dryness. Add 10μl protein sample buffer and boil before loading onto polyacrylamide gel for electrophoresis.

SDS-PAGE

To analyze protein samples based on molecular weight, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used according to the Laemmli method (73). 10% acrylamide mini-gels were run at 150 volts for 45 minutes to 1 hour. Gels were placed in Coomassie Brilliant Blue for 2 hours and destained for at least 2 hours. Protein molecular weight markers from BioRad were used to make the standard curve for size estimations of sample bands.

Fluorescence Studies

Fluorescence studies were carried out at 25°C using a quartz cuvette with a 1cm light path and a 2ml volume. For all fluorescence studies the following settings were used:

Amplifier AC (make sure chopper is on), Energy Mode, Auto Dynode Voltage: coarse = 1, Meter Selector Switch: dynode voltage, Manual Dynode Voltage: Int, adjust Manual Dynode Voltage knob until Dynode Voltage = 0.7 (700 volts), Zero Suppression Coarse knob: off, Response (SEC) Switch: 0.3, Coarse Sample Sensitivity: 0.1. Adjust Fine Sample Sensitivity for each sample.

Recorder X = 50 mv / cm (x1), Y = 50 mv / cm (x1)

All solutions to be read in the fluorimeter must be filtered and degassed. Open sample shutter and adjust sample sensitivity. Zero sample and set recorder pen on the lower left corner of the graph paper. The pen is moved manually for units along the x-axis. For dimerization studies the pen was moved along the x-axis three squares every 3 minutes. The y-axis is moved automatically by the fluorimeter as it reads fluorescence. Points on the graph paper are marked manually by lowering and then raising the pen. Relative fluorescence is calculated by dividing the fluorescence intensity of a 100 % monomer solution by the fluorescence intensity of the sample. Data was corrected for background intensity of the buffer and Raman scatter using a buffer blank and filters.

Fluorescence Analysis of the Dissociation of RT Subunits

The effect of increasing acetonitrile concentrations on subunit interactions of wild type and mutant forms of RT was determined using intrinsic protein fluorescence measurements. 20μl of each sample (0.5μg/μl = 7.5μM) is mixed with varying amounts of 100% acetonitrile and fluorescence buffer to give final concentrations of 6μM RT and either 0,2,4,8,12,16,18, or 20% acetonitrile. The samples were mixed and incubated 10 minutes at room temperature to reach equilibrium. The entire sample is then added to 1.975ml of fluorescence buffer and relative fluorescence intensity recorded. The procedure was carried out in triplicate for wild type RT and for each mutant and the data was averaged and plotted as relative fluorescence on Cricket Graph III.

Fluorescence Analysis of the Association of RT Subunits

To analyze dimerization, the re-association of subunits which have been dissociated with 17% acetonitrile was observed over time. First, 19.5μl of RT (2μg/μl) was mixed with 4μl of 100% acetonitrile and incubated 10 minutes at room temperature to dissociate the subunits. Then the mixture was added to 1.976ml of fluorescence buffer in a quartz cuvette which dilutes the acetonitrile concentration 10-fold and allows the
subunits to re-associate. The intrinsic fluorescence was recorded every 3 minutes for 60 minutes. The procedure was carried out in triplicate for wild type RT and for each mutant and the data averaged. The data was converted to relative fluorescence and plotted using Cricket Graph III.

**Polymerase Activity Analysis of the Dissociation of Subunits**

The effect of increasing acetonitrile concentrations on subunit interactions of wild type and mutant forms of RT was determined using the RT polymerase activity assay. Each RT sample (0.5μg/μl) was diluted 1/10 in enzyme diluent and 1μl removed and added to each of 11 tubes containing varying amounts of 100% acetonitrile and enzyme diluent to give final concentrations of either 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20% acetonitrile in a final volume of 10μl. The samples were incubated 10 minutes at room temperature to reach equilibrium. The entire contents of the tube was added to 40μl of RT polymerase activity reaction cocktail to initiate the reaction and the assay was carried out as described for the RT polymerase activity assay described previously. Each experiment was repeated three times and the data averaged and the results plotted using Cricket Graph III.

**Analytical Gel Filtration Analysis of Dissociation**

Two TSK G3000 SW HPLC gel filtration columns were set up in series at room temperature and equilibrated in a buffer containing 8% acetonitrile by programming the HPLC pump as follows: 84% of reservoir #1 which contains 50mM Tris-Cl pH 7.5 and 100mM KCl, and 16% of reservoir #3 which contains 50% acetonitrile. The high pressure limit was set at 800psi, the flow rate was 0.3ml/min, and the AUFS was set at 0.02. The integrator was programmed the same as it was for protein purification. The sample was prepared by combining 40μl of RT (2μg/μl), 8μl of 50% acetonitrile, and 2μl of buffer (50mM Tris-Cl pH 7.5 and 100mM KCl). The sample was incubated for 10
minutes at room temperature before being injected into the HPLC column. 50μl of sample was injected into a 25μl sample loop.

**RNase H Activity**

Assays for RNaseH activity were performed essentially as described by Moen and coworkers (74). First, make up 750μl of reaction cocktail: 660μl of sterile Milli-Q H₂O, 75μl of 10x annealing buffer, 7.5μl poly(dT) 33.4pmols/μl, and 7.5μl [3H]poly(rA) 32.5pmols/μl. Mix and put 150μl into each of five 0.5ml tubes, which will be for five enzyme RNase H assays. Run annealing program on the thermocycler:

- Step 1 90° C 0 minutes
- Step 2 90° C 3 minutes
- Step 3 60° C 0 minutes
- Step 4 60° C 0 minutes
- Step 5 25° C 60 minutes
- Step 6 25° C 0 minutes

Label five stop tubes for each reaction tube as 0, 15, 30, 45, and 60 minutes, add 800μl of 10% TCA solution to each tube, and place on ice. Start each assay 30 seconds apart. At time 0 for each assay add enzyme, mix, remove a 25μl aliquot and put it in the appropriate stop tube, and place the rest of the reaction mix in a 37°C water bath. At the appropriate times remove 25μl aliquots and add to the stop solution on ice. 5μl of BSA(15mg/ml) was added to each tube after the reactions were stopped. The samples were filtered on nitrocellulose and washed with approximately 10mls of 10% TCA solution. The filters were rinsed with 1-2mls of ethanol and counted in 5mls of scintillation cocktail and counted on a Beckman Liquid Scintillation Counter. The assay was repeated three times for each enzyme and the counts averaged. An assay was run in which no enzyme was added as a negative control. For a positive control, 16U of AMV-RT was added to 150μl RNase H reaction cocktail. For wild type RT and for each mutant RT, 1μl of a 1μg/μl enzyme solution was added to 150μl of RNase H reaction cocktail.

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RESULTS

Growth Curve for New Yeast Strain, XS95-6C

In order to use this strain of *Saccharomyces cerevisiae* for cloning and expression of the mutant forms of RT5, it was helpful to determine at what times different stages of cell growth occur. The growth curve can be divided into three main phases: lag phase, log phase, and stationary phase. Most cloning and gene expression activities are carried out during the log phase. The log phase is further divided into early-log which is characterized by a cell density of < 10⁷ cells/ml, mid-log phase has a cell density of 1 to 5 x 10⁷ cells/ml, and late-log which has a cell density from 5 x 10⁷ to 2 x 10⁸ cells/ml. The following growth curve was obtained by taking OD₆₀₀ measurements of the cell culture as described in Methods over a 44 hour period (see Figure 10). As can be seen in this graph, early log phase occurs at 14 to 20 hours, mid-log at 20 to 25 hours, and late-log at 25 to 33 hours. Transformation of yeast cells is most effective when yeast cells are at the beginning of mid-log phase or 0.5 OD₆₀₀. Yeast cells for plasmid preparations are usually grown to the stationary phase for maximum production of plasmids. The optimal growth phase for expression and synthesis of mutant RT enzyme was determined in another experiment described later.

Isolation of pAB24/RT5 from Yeast and Transformation into *E. coli*

The first step in creating a site-specific mutant of HIV-1 RT using PCR mutagenesis was to obtain template DNA which was pAB24/RT5, the plasmid containing the gene for the wild type enzyme. pAB24/RT5 is a shuttle vector which was transferred to
Figure 10. Growth curve for yeast strain XS95. The graph shows different phases of growth over a 44 hour period in YPD medium. Cell density is quantitated by measuring light absorbance at 600nm using a 1cm plastic cuvette in a Bausch and Lomb spectrophotometer and is expressed as optical density (OD).
for recombinant DNA work because plasmid amplification and preparation is more efficient using E. coli. We used the strain HB101, which is easily transformed and provides large scale growth and production of plasmid. The plasmid was isolated from yeast, and transformed into competent HB101 E. coli cells as described in the Methods section. One colony grew on the selective plate which was used to grow an overnight culture. A plasmid miniprep was carried out on 1.5mls of this overnight culture and the rest was stored in 15% glycerol at -70°C. The isolated plasmid was digested with BamH-1 and Sal-1 restriction enzymes in one reaction, and Hind-3 in another reaction. The presence of pAB24/RT5 was confirmed by the enzyme digest patterns of bands visualized in a 1% agarose gel (see Figure 11). BamH-1 and Sal-1 cut the plasmid into 2 fragments, Hind-3 cut pAB24/RT5 into 6 fragments. The size of the bands on the gel were estimated using a standard curve based on the migration distances of fragments of Lambda DNA BstE-2 digest.

More of the transformed HB101 E. coli cells were grown from -70°C glycerol stock cultures and several minipreps carried out in order to obtain enough plasmid DNA to serve as template for PCR mutagenesis. A total of 50μl of a 12μg/μl solution of purified plasmid DNA was obtained, which was more than enough for PCR procedures since only 0.1 to 250ng of template DNA were needed for each reaction. Large amounts of plasmid DNA, pAB24/RT5, were needed later for putting the mutated fragment of DNA back into the cloning vector.

**PCR Mutagenesis of RT5**

A procedure for creating a single base pair substitution at amino acid position L289 of HIV-1 RT using PCR mutagenesis was designed and is summarized in Figure 5 of the Methods section. In order to determine if the first set of PCR reactions for making fragment A and fragment B worked, 5μl aliquots from each of the 100μl PCR reaction mixes were analyzed on a 1% agarose gel. Figure 12 shows PCR fragments A and B.
Figure 11. 1% agarose gel electrophoresis showing wild type plasmid isolated from transformed HB101 *E. coli* and digested with restriction enzymes. Numbers represent the size of fragments in kilobases. a) standard size marker: Lambda DNA BstE digest, b) and c) pAB24/RT5 digested with BamH-1 and Sal-1, d) pAB24/RT5 digested with Hind-3.
Figure 12. Preparative 1% agarose gel showing PCR fragments. a) PCR fragment A, b) PCR fragment B. The fragments are produced in separate PCR experiments and must be isolated from the gel, purified and combined in a third PCR experiment to produce the PCR mutated fragment C. Numbers represent fragment sizes in kilobases.
Fragment A is synthesized using primers 1 and 2 (for making mutant L289R) and primers 1 and 4 (for making mutant L289P). Fragment B is synthesized using primers 3 and 6 (for making mutant L289R) and primers 5 and 6 (for making mutant L289P). All of the A and B PCR products were isolated from the PCR reaction mixture by running on a preparative agarose gel using low melting temperature agarose. The preparative gel was run using large sample wells as shown in Figure 12. Once the A and B fragments were isolated and purified they were combined in a second PCR reaction using only the outside primers 1 and 6, which resulted in the formation of fragment C. A comparison of the different sizes of all 3 fragments can be seen in Figure 13. Fragment C, which contains the desired base pair substitution, was isolated from the PCR reaction mix by running on a preparative gel as described for fragments A and B. Once a large quantity of purified PCR mutated fragment C was obtained, it was digested with AlwN-1 and Sal-1 to produce the necessary cohesive ends for placing the mutated fragment back into the expression vector (Figure 14).

Placing Mutant Fragment C into Expression Vector

The procedure for placing mutated fragment C into pAB24 is summarized in Figure 9 of the Methods section. The first step of digesting pAB24/RT5 with BamH-1 and Sal-1 and purifying the DNA fragments was repeated many times to obtain large amounts of BamH-1/Sal-1 large and small fragments (Figure 15). All BamH-1/Sal-1 large fragment samples were isolated, purified, pooled, quantified, and stored at -20°C. The BamH-1/Sal-1 small fragment samples were also isolated, purified, and quantified, but then they were digested with AlwN-1 and Pvu-2. The double digest was run on a 1% agarose gel and the 1,025 base pair BamH-1/AlwN-1 fragment was isolated and purified. The ligation step was done in two steps, and the best conditions and concentrations of fragments was determined empirically. The BamH-1/AlwN-1 fragment was first ligated to the mutant AlwN-1/Sal-1 fragment to produce a BamH-1/Sal-1 fragment which contained the mutation. Then the BamH-1/Sal-1 large fragment containing the body of the plasmid was
**Figure 13.** 1% agarose gel showing all three PCR fragments: a) PCR fragment A, b) PCR fragment B, and c) PCR fragment C which is made by combining fragments A and B. Numbers indicate the size of each fragment in kilobases.
Figure 14. Preparative 1% agarose gel for isolating PCR mutated fragment C which has been digested with AlwN-1 and Sal-1 restriction enzymes. Numbers indicate fragment size in kilobases. The fragments visible in lanes a) and b) were cut out and purified by phenol extraction and ethanol precipitation.
Figure 15. Preparative 1% agarose gel showing multiple BamH-I/Sal-I double digests of pAB24 in lanes a-d. Numbers are the fragment sizes in kilobases. Both large and small fragments are isolated from the gel, purified, and used for inserting mutated fragment C into pAB24 expression vector.
added to this same ligation mixture and incubated again to produce closed, circular plasmid DNA containing the mutated RT gene. For each ligation step the fragments being ligated possessed overhangs with different restriction sequences at each end so they could combine in only one possible direction. The ligation mixture was directly used to transform HB101 E. coli cells which were spread onto LB + Ampicillin plates (Figure 16). The ligation/transformation experiment for each mutant produced only 1 to 4 colonies per plate and 3ml overnight cultures were grown from each colony in LB + Ampicillin broth. 1.5mls of the overnight culture was used to make glycerol stock cultures and the rest of the overnight culture was used to do a mini-plasmid prep for restriction enzyme analysis using BamH-1 and Sal-1 to verify that pAB24/RT was present. The successfully cloned, mutated RT gene was then ready to be sequenced to verify that the desired site-specific mutation had been produced.

**DNA Sequencing**

The region of the RT gene that was sequenced was the region that was manipulated by PCR mutagenesis to produce the site-specific mutant. This sequence corresponds to the PCR fragment C which is bound by AlwN-1 and Sal-1 restriction sites. The same primers that were used for PCR mutagenesis were used for DNA sequencing using Promega's Silver Sequence method. Each mutant was sequenced over the entire 1,040 bases which were PCR amplified, and for each sequencing experiment an average of 200 bases could be read. Sequences that were questionable were repeated and no other mutations were found. Sequences showing the desired mutations are shown in Figures 17 and 18. As can be seen in Figure 17 the T has been changed to a G to produce a codon for arginine instead of leucine for the L289R mutant. Figure 18 shows that the T has been changed to a C to produce the codon for proline for the L289P mutant.
Figure 16. Photograph of an LB + ampicillin selective plate with three colonies of HB101 *E. coli* transformed with the plasmid, pAB24/RT5M1, containing the mutated RT gene. Only the bacteria which contain this plasmid will grow on the selective plate. Further confirmation that the correct plasmid is in the HB101 *E. coli* cells is obtained by growing an overnight culture from one of the colonies, doing a plasmid prep, and digesting the plasmid with BamH-1 and Sal-1.
**Figure 17.** Photograph of a DNA sequencing gel showing the T to G mutation which will result in the amino acid change, L289R. The sequence shown on the left hand side of the picture is read from the bottom, which is the 5' end, to the top, which is the 3' end of the sequence.
Figure 18. Photograph of a DNA sequencing gel showing the T to C mutation which will result in the amino acid change, L289P. Part of the sequence containing the mutation is shown on the left and is read from the bottom (5'end) to the top (3'end).
Yeast Transformation

The yeast strain XS95 was successfully transformed with both mutant plasmids using the spheroplast method (70). Several different quantities of DNA were used, and all were successful. A critical factor was the optical density of the yeast cell culture that was to be transformed, which should be no more than 0.5 OD<sub>600</sub>. Also, sterile conditions must be maintained throughout the procedure and spheroplasts must be treated very gently. Yeast colonies that grew embedded in the selective regeneration agar formed star shaped colonies while those that grew on top of the agar formed the normal smooth, round colonies (see Figure 19). Several colonies were picked and overnight cultures grown in Leu minus rich medium. Half of the overnight culture was stored as a glycerol stock and the other half analyzed by restriction enzyme digest with BamH-1 and Sal-1 (see Figure 20). Several 1 liter cultures of XS95 yeast containing the mutated pAB24/RT were grown for large scale protein preparations.

Yeast Expression Experiment

This experiment was carried out to determine if and when the yeast strain XS95 carrying mutated plasmid would express the RT gene. For yeast expression of the RT gene to be initiated, the GAPDH promoter must be turned on by a glucose deficiency. Decreased levels of glucose in the YPD medium occurs as the yeast cells grow and divide and metabolize glucose. As glucose levels decrease, the GAPDH promoter is activated and expression of RT is amplified. The data summarized in Table 1 shows that over time, as the OD<sub>600</sub> of the culture increased, the glucose levels decreased and RT activity in protein extracts increased. The optical density of the cell culture began to slow down after 14 hours which is the same time that glucose levels dropped to undetectable levels (see Figure 21), however, RT activity continued to increase (Figure 22). As can be seen in Figure 23, protein concentration measurements parallel RT activity and reflect the synthesis of large amounts of protein. After 18 hours of growth, RT activity decreased slightly indicating
Figure 19. Photograph of a leucine minus selective plate showing colonies of yeast strain XS95 transformed with the mutated plasmid, pAB24/RT5M1. Several of the colonies were picked for growing up overnight cultures for plasmid preps which were analyzed by restriction enzyme digests to confirm that the correct plasmid was present in the yeast.
Figure 20. Photograph of a 1% agarose gel showing plasmid from transformed yeast digested with BamH-I and Sal-I in lanes d and e. Lanes a and b show undigested plasmid and lane c shows the Lambda DNA marker.
Table 1. Mutant RT expression experiment in yeast strain XS95. Yeast cells transformed with the mutated plasmid are grown for 24 hours in YPD medium. Samples of the culture are taken every hour to measure cell density, glucose level, pH, protein concentration, and RT activity.
## Expression of L289R Mutant RT in Yeast Strain XS95

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Figure 21. Mutant RT expression experiment in yeast strain XS95. The cell culture is grown in YPD medium for 24 hours and samples analyzed every hour. The graph has a double Y-axis showing glucose concentration and cell density over culture incubation time. Glucose (mg/dl)  Cell Density (OD\textsubscript{600})
Figure 22. Mutant RT expression experiment in yeast strain XS95. The cell culture is grown in YPD medium for 24 hours and samples analyzed every hour. The graph has a double Y-axis showing glucose concentration and RT Activity over culture incubation time.

- Glucose (mg/dl)  
- Activity (nmol/ml)
Figure 23. Mutant RT expression experiment in yeast strain XS95. The cell culture is grown in YPD medium for 24 hours and samples analyzed every hour. The graph has a double Y-axis showing protein concentration and RT Activity over culture incubation time.

- Protein Concentration (µg/µl)
- Activity (nmol/ml)
either a drop in expression of RT or destruction of the protein by yeast proteases. Since this yeast strain is not protease negative, it may synthesize proteases in response to the production of a non-native protein, which could make RT isolation difficult or impossible. However, after 3 more hours the RT activity began to rise again, which may mean that RT synthesis became greater than protease degradation, or that RT was being converted to heterodimer, the mature form of the enzyme. For large scale protein preps the XS95 yeast should be grown 25 hours before being harvested for protein purification. Protease inhibitors must be included throughout the purification procedure and all solutions containing RT must be kept ice cold to further inhibit protease activity.

Comparative Purification of Mutants on P11 Column:

Whatman P11 cellulose phosphate contains an ester-linked phosphate functional group which acts as a cation exchanger. The cellulose phosphate will bind or release molecules depending on the charge of the molecule and the ionic strength of the eluant buffer. P11 also acts as a specific adsorbent for enzymes such as DNA polymerases that have a high affinity for phosphate ions. The phosphate groups on the P11 column may resemble the phosphate group on dNTPs, binding to the dNTP substrate binding site on the enzyme. When cell lysate containing RT is passed through the P11 column using standard RT buffer (RTB), which contains 50mM KCl, RT will bind to the phosphorylated cellulose. The column is washed with 50mM KCl RTB to remove any molecules not bound to the cellulose phosphate. When a buffer salt gradient is passed through the column RT will be released from the cellulose phosphate at a specific salt concentration. In a 50-800mM KCl gradient in RTB, wild type RT elutes in 300-400mM KCl.

The purification procedure was repeated three times for each mutant under the same conditions in order to observe any differences in elution characteristics which may indicate changes in molecular charge of the enzyme. Each experiment started with growth and expression of 1 liter of XS95 cells containing the mutated RT gene. Protein was
precipitated from the cell culture using ammonium sulfate, dialyzed to remove salt, and loaded in a volume of 200mls onto the P11 column. While running a 50-800mM KCl gradient in RTB with a pump flow rate of 0.5ml/minutes, samples were collected in a fraction collector set at 16 minutes /tube. Fractions were assayed for RT polymerase activity and protein concentration and plotted versus elution volume using Cricket Graph III (Figures 24-31). From this graph it could be determined at what volume the enzyme elutes. Then, by plotting the elution volume versus the salt concentration, the salt concentration in which each mutant elutes could be determined. The results for the three P11 preps of mutant L289R are shown in Figures 24-26. Figure 27 shows that L289R elutes in an average of 118mM to 245mM KCl in RTB. The results of the three L289P P11 preps are shown in Figures 28-30. Figure 31 shows that mutant L289P elutes in an average of 121mM to 247mM KCl in RTB. Both mutant enzymes eluted at lower KCl concentrations than wild type RT which elutes in 300-400mM KCl, however, the elution characteristics of the two mutants were similar to each other. These results indicate that the mutations may have affected the structure of the enzymes and dimer stability so that the mutants elute from the P11 column in a lower ionic strength solution than wild type enzyme.
Figure 24. Comparative purification of mutants on the P11 column: Mutant L289R prep #1. The graph shows activity as nmol/ml (●—●) and protein concentration as absorbance at 595nm (○—○). Peak RT activity occurs at 145mM KCl. The mutant RT elutes in a range of 130 to 230mM KCl.
Figure 25. Comparative purification of mutants on the P11 column: Mutant L289R prep #2. The graph shows activity as nmol/ml (-----) and protein concentration as absorbance at 595nm (.....). Peak RT activity occurs at 163mM KCl. The mutant RT elutes in a range of 135 to 258mM KCl.
Figure 26. Comparative purification of mutants on the P11 column: Mutant L289R prep #3. The graph shows activity as nmol/ml (---) and protein concentration as absorbance at 595nm (••••••••). Peak RT activity occurs at 97mM KCl. Mutant RT elutes in a range of 88 to 248mM KCl.
Figure 27. Comparative purification of mutants on the P11 column: Mutant L289R, average of three preps. The graph shows the linear salt gradient versus elution volume. Mutant L289R elutes in an average of 118-245mM KCl.
Figure 28. Comparative purification of mutants on the P11 column: Mutant L289P prep #1. The graph shows activity as nmol/ml (—□—) and protein concentration as absorbance at 595nm (—○—). Peak RT activity occurs at 180mM KCl. The mutant RT elutes in a range of 150-225mM KCl.
Protein Concentration (Abs 595nm)

Elution Volume (mls)

Activity (mmol/ml)
Figure 29. Comparative purification of mutants on the P11 column: Mutant L289P prep #2. The graph shows activity as nmol/ml (- - -) and protein concentration as absorbance at 595nm (• • •). Peak RT activity occurs at 88mM KCl. Mutant L289P RT elutes in a range of 78-239mM KCl.
**Figure 30.** Comparative purification of mutants on the P11 column: Mutant L289P prep #3. The graph shows activity as nmol/ml (---) and protein concentration as absorbance at 595nm (--O--O--). Peak RT activity occurs at 154mM KCl. Mutant L289P RT elutes in a range of 135-276mM KCl.
Figure 31. Comparative purification of mutants on the P11 column: Mutant L289P, average of three preps. The graph shows the linear salt gradient versus elution volume. Mutant L289P elutes in an average of 121-247mM KCl.
Completion of Mutant Purification

After the initial P11 purification step was completed and the fractions containing the RT peak pooled, a 40ml average sample volume was obtained. The samples from two out of the three preps for each mutant were concentrated down to 2mls and stored at -20°C in the non-defrosting freezer. The other 40 ml samples, 12.5mg for the L289R mutant and 35mg for the L289P mutant, from each P11 prep were loaded onto the Affi-Gel Blue column (see Figures 32 and 33). After pooling, desalting and concentrating fractions from each mutant prep, 3.1mg in 1.5ml was obtained for the L289R mutant and 5.3mg in 2ml was obtained for the L289P mutant. These samples were then passed through the Superose 12 gel filtration column in 25μl injections and the fractions collected, pooled, and concentrated (see Figures 34 and 35). For the L289R mutant, 500μl of a 1.8μg/μl sample was obtained. For the L289P mutant, 500μl of a 2μg/μl sample was obtained. The retention times of peaks observed coming off the Superose 12 column when L289R was injected showed a standard deviation of 4.31 minutes with an average of 85.97 minutes. Using the standard curve shown in Figure 36 to calculate the estimated molecular weight of this peak, the sample is approximately 98,000 Da. This value is close to 99,000 which would be the average molecular weight of a mixture of monomers (66,000 Da) and dimers (132,000 Da), which is what we expect to observe since the Superose 12 column cannot resolve monomers from dimers. The range of retention times observed for the L289R mutant, 81.66 to 90.28, overlaps the range observed for the L289P mutant which was 82.31 to 90.15 minutes. The average retention time for the L289P mutant was 86.23 minutes which equals approximately 96,000Da based on the standard curve. This estimated molecular weight value is slightly less than the value observed for L289R which may mean that there is a greater percentage of monomers present in the L289P sample.
Figure 32. SDS-PAGE of the mutant L289R prep showing pools from Affi-Gel Blue column. Fractions were collected from the Affi-Gel Blue column and assayed for RT activity and protein concentration. Fractions containing peak RT activity were pooled, concentrated, and desalted. Lane d shows pool #2 which has the greatest RT activity; lanes a and g are molecular weight markers; lane b is crude extract; lane c is pool #1; lane e is pool #3; and lane f is pool #4. Numbers represent apparent molecular weight in kilodaltons.
Figure 33. SDS-PAGE of mutant L289P prep showing fractions collected from the Affi-Gel Blue column. 500μl of each fraction was TCA precipitated, resuspended in 15μl of sample buffer, and run on a 10% polyacrylamide gel. Lane a shows molecular weight markers; lanes b-h are fractions 6, 8, 10, 19, 22, 24, and 25. Numbers represent the apparent molecular weight in kilodaltons. The greatest amount of mutant RT is in fraction #10, lane d.
Figure 34. SDS-PAGE analysis of mutant L289R purified by the Superose 12 column. Fractions collected from the column were analyzed for RT activity and samples containing peak activity were pooled and concentrated. 10μl of this pool was run on the 10% polyacrylamide gel in lane b. Lane c shows wild type RT and lane a shows the molecular weight markers. The numbers are the apparent molecular weights in kilodaltons.
Figure 35. SDS-PAGE analysis of mutant L289P purified by the Superose 12 column. Fractions collected from the column were analyzed for RT activity and samples containing peak activity were pooled and concentrated. 100μl of this pool was TCA precipitated, resuspended in sample buffer, and run on the 10% polyacrylamide gel in lane b. Lane c shows wild type RT and lane a shows the molecular weight markers. The numbers are the apparent molecular weights in kilodaltons.
**Figure 36.** Graph of the protein standard curve for the Superose 12 FPLC gel filtration column. 25μl of BioRad gel filtration standards were injected onto the column and the retention time for each protein peak was plotted against the log of the molecular weight of the protein peak. Point A = 158KDa, point B = 44KDa, point C = 17KDa, and point D = 1.35KDa. The equation for the standard curve was calculated by Cricket Graph III to be: 
\[ y = -0.039x + 8.344 \]
and was used to determine the expected retention time for mutant RT based on the molecular weight of the monomer and dimer forms of RT.
The two mutant RT samples collected from the Superose 12 column were injected onto tandem TSK gel filtration columns for final purification. The RT peak fractions collected from this column were pooled and concentrated as before. The final yield of purified protein was 250μl of the L289R mutant at 1.08μg/μl, and 500μl of the L289P mutant at 1.02μg/μl. The samples were run on a 10% SDS-PAGE gel (see Figure 37) which was scanned by a densitometer to determine the percent purity of each mutant RT sample.

Densitometric Gel Scan

Densitometric scans of the gel in Figure 37 were made on a GelScan XL (2.1) densitometer with the permission of Dr. Castora at Eastern Virginia Medical School. Quantitation of the peaks was made by cutting out the peaks and weighing them. The scan of the standard markers in lane #4 of the gel is shown in Figure 38. Wild type RT5 makes up 81% of the total protein in sample lane #1 as measured by the scan shown in Figure 39. The L289R mutant RT in lane #2 makes up 84% of the protein in this sample as measured by the scan shown in Figure 40. Mutant L289P RT makes up 66% of the total protein in this sample as determined from the scan shown in Figure 41. These values must be taken into consideration when making calculations involving protein concentration. For example, specific activity is based on units of RT activity divided by the quantity RT in the sample. The amount of RT is equal to the protein concentration determined by the Pierce protein assay multiplied by the percent of RT measured by the densitometer.
Figure 37. SDS-PAGE analysis of different forms of RT after final purification step. Lane a is wild type RT, lane b is mutant L289R, and lane c is mutant L289P. Molecular weight markers are shown in lane d. Numbers represent the apparent molecular weight in kilodaltons.
Figure 38. Densitometric gel scan of molecular weight markers in lane #4 of the SDS-PAGE gel shown in figure 37.
Figure 39. Densitometric scan of wild type RT5 in lane #1 of the SDS-PAGE gel shown in Figure 37. Peak sizes were quantitated by cutting them out and weighing them. The RT5 peak was determined to make up 81% of the total protein in the sample.
Figure 40. Densitometric scan of mutant L289R in lane #2 of the SDS-PAGE gel shown in figure 37. This mutant was determined to make up 84% of the total protein in the sample.
Figure 41. Densitometric scan of mutant L289P in lane #3 of the SDS-PAGE gel shown in figure 37. This mutant was found to make up 66% of the total protein in the sample.
Effect of RT Concentration on RT Polymerase Activity

In theory, as enzyme concentration is increased, the activity of the enzyme is expected to increase in a linear relationship. However, in practice, the relationship is linear only at a certain enzyme concentrations. In order to determine the best enzyme concentration for activity assays several different dilutions were prepared using 1μg/μl samples of each mutant RT. As can be seen in Figure 42, dilutions in the range of 1/120 to 1/240, which have final concentrations of 4.2ng/μl to 8.3ng/μl, were in the linear region of the curve for both mutant forms of RT. Mutant RT concentrations in this range will be used for all experiments involving RT polymerase activity.

Specific Activity of RT Mutants

The specific activity of RT is defined as the nmoles of [3H]dTTP incorporated per μg of RT. For determining the specific activity of each mutant RT, the RT polymerase activity assay was carried out using a dilution of enzyme in the linear range of RT activity as determined above. The assay was carried out in triplicate for each enzyme sample and the nmoles of substrate incorporated per μg of enzyme determined. The specific activity measurements must be corrected by multiplying them by the percent of RT in each enzyme sample as determined by the densitometric scans described above. The results are summarized in Figure 43 and show that mutant L289P has significantly lower specific activity than wild type RT, but is not significantly different from mutant L289R. Mutant L289R also appears to have lower activity than wild type RT but when error is taken into consideration it is not significantly different. The lower specific activity of the L289P mutant may be caused by impurities in the sample since it was determined to be only 66% of the total protein in the sample. These results raise some interesting questions about how these two mutants compare with each other as well as how they compare with wild type enzyme. These questions will be answered by further comparisons of enzyme behavior.
Figure 42. Experiment to determine the enzyme concentrations which have a linear relationship with enzyme activity. Panel A is a graph of the activity of mutant L289R at different concentrations. Dilutions in the range of 1/120 to 1/240 were in the most linear region of the curve. This means that RT concentrations from 4.2ng/μl or 63.6nM to 8.3ng/μl or 125.8nM should be used for activity assays. Panel B is a graph of the activity of mutant L289P at different concentrations and the results were the same as for L289R.
Figure 43. Specific activity determinations for RT mutants L289R and L289P. The specific activity of the mutants is compared to wild type RT which has been previously determined (11).
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<tr>
<td>Mutant L289R</td>
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<tr>
<td>Mutant L289P</td>
<td>8.39 ± 1.12</td>
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Fluorescence and the Dissociation of Subunits in Increasing Concentrations of Acetonitrile

Earlier studies have already shown that fluorescence can be used to analyze dimerization of HIV-RT (32). The heterodimeric enzyme contains a total of 37 tryptophan residues, 19 on the p66 subunit and 18 on the p51 subunit. As the molecule undergoes conformational changes, some of the tryptophans either become buried or exposed resulting in a change in fluorescence intensity of the molecule and the solution as a whole. Divita and coworkers found that when looking at the dissociation and reassociation of RT subunits there was a significant (25%) change in the fluorescence intensity which allowed the use of this method to do kinetic studies of dissociation and dimerization (32). Previously, only analytical gel filtration (29, 40), analytical ultracentrifugation (33, 40), and circular dichroism spectroscopy (34, 40) have been used to study association and dissociation of RT subunits. The fluorescence method has been shown to be as reliable as these other methods and even better for studying dissociation because it can measure changes that occur within a few seconds (32). The fluorescence method of analyzing subunit interactions has also been shown to directly correlate with RT polymerase activity. Acetonitrile is an organic solvent which, at a concentration of 17% to 20%, has been shown to dissociate the dimer into monomers without causing complete unfolding of the subunits (29, 32, 75).

The dissociation of subunits in increasing concentrations of acetonitrile was compared for wild type RT and the two RT mutants, L289R and L289P. The results, shown in Figure 44, are three sigmoidal curves similar to the curve obtained by Divita’s group (32). The two RT mutants created in our lab showed a significantly different dissociation pattern from wild type RT in the region between 6 and 16% acetonitrile. Both mutants appear to dissociate into monomers at lower concentrations of acetonitrile than wild type RT. The wild type RT was 50% dissociated into monomers at approximately 11.5% acetonitrile whereas both of the mutants were 50% dissociated at 7.5%. All three
Figure 44. Fluorescence studies showing the dissociation of subunits in increasing concentrations of acetonitrile. Relative fluorescence is expressed as a fraction of the observed intrinsic fluorescence of the sample divided by the fluorescence of 100% monomeric RT. As the percentage of acetonitrile in the sample increases, more of the subunits dissociate resulting in increased fluorescence. The results for all three forms of RT are shown in the graph.

- Wild Type RT5
- Mutant L289R
- Mutant L289P
samples of RT were completely dissociated into monomers at 18% acetonitrile. The two mutants differed slightly from each other at concentrations above 8% acetonitrile. Mutant L289R maintained a slightly higher percentage of dimers at higher concentrations of acetonitrile than mutant L289P which was completely dissociated at 16% acetonitrile.

**RT Polymerase Activity and the Dissociation of Subunits in Increasing Concentrations of Acetonitrile**

A previous study has shown that RT subunit dissociation can be directly correlated with RT intrinsic fluorescence as well as with RT polymerase activity (32). The RT polymerase activity assay has an advantage over fluorescence in that it requires a much smaller quantity of enzyme. However, fluorescence is important because, although RT activity is affected by subunit interactions, fluorescence is a direct measurement of subunit interactions. Therefore, it was desirable to correlate the fluorescence observations that the mutants appeared to dissociate more easily than wild type RT, and also that L289P may dissociate slightly more readily than mutant L289R with effects on RT activity. The RT polymerase activity assay was used for another comparative study of the dissociation of RT subunits in different concentrations of acetonitrile. The samples were set up in different concentrations of acetonitrile just as they were for the fluorescence study, plus three additional concentrations were used. The preparation of the sample mixture was timed so that the enzyme would be incubated with the acetonitrile for 10 minutes before starting the activity assay to allow the effect of acetonitrile on subunit interactions to reach equilibrium. Figure 45 shows that as the enzyme was incubated in higher percentages of acetonitrile, the activity of the enzyme decreased due to dissociation of the subunits. This graph shows that both mutants are significantly different from wild type RT in the range of 4 to 14% acetonitrile and also from 16 to 20% acetonitrile. As seen in the analogous fluorescence experiment, the two mutants are closer to each other in their dissociation pattern than to wild type RT. Also, as seen before, mutant L289P shows a greater deviation from wild
Figure 45. RT polymerase activity analysis of the dissociation of subunits in increasing concentrations of acetonitrile. As the percentage of acetonitrile in the sample increases, the activity decreases due to the dissociation of the subunits. The results for all three forms of RT are shown in the graph.

---●--- Wild Type RT5  ---◇--- Mutant L289R  ---×--- Mutant L289P
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type RT behavior than does mutant L289R. These results support the observations from the previous experiment that both mutants dissociate into monomers more easily than wild type RT, and that mutant L289P dissociates into monomers more readily than mutant L289R.

Analytical Gel filtration Analysis of the Dissociation of RT Subunits in 8% Acetonitrile

Tandem HPLC gel filtration columns were used to observe the shift in percentage of monomers versus dimers when RT is incubated in acetonitrile. This experiment supports the fluorescence and activity observations that acetonitrile causes the RT subunits to dissociate without denaturing them. The fluorescence studies showed a 50% dissociation of subunits for both mutant enzymes in 8% acetonitrile, so this concentration of acetonitrile was used for the gel filtration analysis. The RT sample was incubated in acetonitrile as described for the fluorescence and activity assays to give a final concentration of 8% acetonitrile. Figure 46A shows the gel filtration chromatogram for mutant L289R that has not been incubated in acetonitrile. The dimeric form of the enzyme, p66/p66, elutes at 77.02 minutes and the monomeric form, p66, elutes at 87.76 minutes. Gel filtration analysis of mutant L289R that was incubated in buffer without acetonitrile shows that 47% of the enzyme was in dimeric form and 53% was monomeric. It is not surprising that the enzyme is slightly more monomeric than dimeric. Rowley and coworkers (27) found purified p66 RT to be an equilibrium mixture of monomers and dimers, and that p66 from frozen stock solutions contained only 36% to 39% dimers. The mutant L289R which eluted from the gel filtration column after 77 minutes had been at room temperature for a total of 87 minutes in a buffer that did not contain glyceral or detergents which are normally used to help stabilize the protein. The buffer which contained only 50mM Tris-Cl pH 7.5 and 100mM KCl was used for the HPLC gel filtration studies because it resulted in better resolution of monomer and dimer peaks. Figure 46B shows mutant L289R that has been
Figure 46. HPLC gel filtration analysis of mutant L289R. Panel A: chromatogram of mutant L289R eluted in 50mM Tris-Cl pH 7.5/100mM KCl, no acetonitrile is present in the elution buffer or in the sample. 47% of the mutant RT elutes in the dimer peak at 77 minutes and 53% elutes in the monomer peak at 87.8 minutes. Panel B: chromatogram of mutant L289R which has been incubated in and eluted in 8% acetonitrile buffer. The dimer peak now makes up only 25% of the enzyme and 75% is monomeric, indicating that 47% of the dimers dissociated into monomers.
incubated in 8% acetonitrile. This chromatogram show that the dimer peak makes up only 25% of the enzyme so that 75% is monomeric. These results demonstrate that in the presence of 8% acetonitrile almost half of the dimerized enzyme dissociates into monomers. As can be seen in this chromatogram, both monomer and dimer peaks eluted 3 minutes later than enzyme which had not been incubated in acetonitrile. This shift may be due to changes in the 3-dimensional structure of the protein caused by the acetonitrile even in subunits that remained dimerized. To confirm that both peaks contained the enzyme, the fractions containing these peaks were collected and analyzed by SDS-PAGE as shown in Figure 47.

**Fluorescence Studies of the Re-Association of RT Subunits After Dissociation with 17% Acetonitrile**

In order to determine if the mutations had any effect on the ability of the subunits to dimerize, the samples were first completely dissociated with 17% acetonitrile and then observed over time for changes in fluorescence as the subunits re-associated. This process of re-association has been shown to occur the same way as subunits that have been separately purified and are combined for the first time without having ever been exposed to acetonitrile (29). This method of observing association of subunits, or dimerization, using fluorescence has already been used to analyze catalytic rates of association for wild type RT (32). For this experiment the association rate of wild type RT was compared with the association rate of mutants L289R and L289P over a period of 1 hour as shown in Figure 48. As time progresses from 0 to 60 minutes, the relative fluorescence of all three samples decreases, indicating that the subunits of the enzyme are forming dimers. The graph shows that all three samples take 50 to 55 minutes to completely dimerize. The rate of association for each enzyme sample was calculated from the slope of each sample curve and is expressed in Table 2 as relative fluorescence units per minute. The graph in Figure 48 shows there are several points on the wild type RT curve that are significantly different.
Figure 47. Photograph of the silver stained, TCA precipitated fractions from HPLC gel filtration of mutant L289R. The gel shows that a 66KDa protein is in both peak fractions.
Figure 48. Fluorescence analysis of the re-association of subunits after dissociation with 17% acetonitrile. After initial dissociation of subunits, the concentration of acetonitrile was reduced 10 fold by dilution with fluorescence buffer which allows the subunits to reassociate to form dimers. The association rates are compared over a period of one hour. Panel A shows mutant L289R compared with wild type RT5. Panel B shows mutant L289P compared with wild type RT5.

- Wild Type RT5
- Mutant L289R
- Mutant L289P
Table 2. Association rates of wild type RT, mutant L289R, and mutant L289P. Rates were calculated from the data of fluorescence analysis of the re-association of subunits over time.
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<tr>
<td>Mutant L289R</td>
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</tr>
<tr>
<td>Mutant L289P</td>
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</table>
from both mutant RT curves. This indicates that wild type RT is associating more rapidly than either of the two mutants. It also appears that there may be a slight difference between the two mutants during the early stages of the dimerization process. When the association rate was calculated based on the slope of the line, the calculations support these observations that wild type RT associates faster than either mutant with an association rate of 0.017 relative fluorescence units per minute, and that mutant L289R, which associates at 0.018 fluorescence units per minute, associates faster than mutant L289P which has a rate of 0.019 fluorescence units per minute.

**Effect of L289 Mutations on RNase H Activity**

The mutants were compared to wild type RT in their ability to degrade the RNA of the tritiated RNA-DNA hybrid as shown in Figure 49. A negative control sample in which no enzyme was added was run along with the HIV-1 RTs. AMV RT was run as a positive control. As time progressed, the RNA in the samples which received RT enzyme was degraded resulting in decreased CPMs, whereas the control sample, which did not receive any enzyme, did not show a decrease in CPMs. All samples which received enzyme were degraded to background levels after 60 minutes. Mutant L289P was not significantly different from mutant L289R in RNase H activity, and neither mutant was significantly different from wild type RT in this activity.
Figure 49. RNase H activity assay of mutant RTs and wild type RT plus a negative control (no enzyme) and a positive control (AMV RT). Counts per minute (CPM) indicate the amount of DNA-RNA hybrid that is trapped on the nitrocellulose filters because it was not degraded by RT RNase H activity.

L289P ---●---  L289R ---○---  RT5 ---●---  AMV RT ---△---

No Enzyme ---+---
DISCUSSION

Two HIV-1 RT mutants have been created using PCR mutagenesis and recombinant DNA techniques. These mutants were expressed in yeast and purified using a cation exchange column, an affinity column, an FPLC gel filtration column, and an HPLC gel filtration column. The presence of RT in the purified sample was confirmed using RT activity assays and SDS-PAGE. The two mutants were compared to each other and to wild type RT in terms of specific activity, subunit dissociation, and subunit association using RT polymerase activity assays, fluorescence studies, and analytical gel filtration. Both mutants showed less specific activity than wild type RT, both mutants dissociated into monomers more easily than wild type RT, and both mutants were slower to associate to form dimers than wild type RT. In addition, the two mutants differed from each other in each of these activities. The L289P RT mutation had a greater affect on subunit interactions than the L289R mutation. Consequently, the L289P mutant dissociated more quickly in the presence of acetonitrile and was slower to re-associate once the acetonitrile was diluted to a non-denaturing concentration than mutant L289R. These results raise the following questions: 1) why did the mutations affect dimerization only partially and not cause them to completely dissociate? 2) why did the mutation L289P have a greater affect on subunit interactions than the L289R mutation? and 3) how do these results compare to the results of other researchers who also made mutations in this region? In order to answer these questions it is helpful to go back and consider why these mutations were made.

This research was undertaken because we wanted to learn more about where and how subunit interactions take place. The mutations that were created were chosen because

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they changed amino acids which were believed to be directly involved in dimerization. Previous research had narrowed down the possible regions involved in subunit interactions to three different locations, all of which were believed to lie in a hydrophobic interface which was not accessible to monoclonal antibodies when the subunits were dimerized (39). The first region was from amino acid 230 to 300 (39), which includes a leucine repeat sequence (38). The leucine repeat sequence in this region was of particular interest because it is very similar to the leucine zipper, a protein-DNA binding motif which enables either two proteins or two subunits of a protein to bind together and at the same time bind DNA. Most of these DNA binding proteins are DNA transcription factors, but it is also a logical binding method for a DNA polymerase dimer in which the subunit binding is known to involve hydrophobic interactions. It also makes sense that, in the case of RT, the protein-DNA binding should be flexible enough to allow the complex catalytic activity of DNA polymerization and RNA degradation. This would explain why the leucine repeat sequence in RT was not a strictly conserved leucine zipper. In a strict leucine zipper motif, two α helices, one from each monomer, are joined by hydrophobic interactions of leucines at every eighth residue on each subunit (77). HIV-1 RT differs from the classical leucine zipper in that it has a threonine in place of one of the leucines and it has a proline in the middle of the motif. Goel and coworkers, as well as my own sequence comparison studies, found the leucine repeat sequence to be conserved among different isolates of HIV-1 RT (40). The second region included amino acids 350 to 428 (39) which contains a large number of tryptophan residues which may also be involved in subunit interactions because they have hydrophobic side chains (75). This theory was further supported by the fluorescence studies of Divita and coworkers (32) who observed a 25% increase in intrinsic fluorescence as dimers dissociated into monomers due to the exposure of tryptophan residues. The third region included amino acids around 540 which is in the RNase H domain (75). This region has been shown to be involved in the interaction between the RNase H domain and the polymerase domain (76). The RNase H region around 540
contains two metal binding sites and it has been shown that the presence of Mg$^{2+}$ increases the association rate of the heterodimer by 100 fold (32). It was suggested that binding of magnesium may result in a conformational change which favors dimerization (32).

The research described in this paper was undertaken because it was theorized that the region between amino acids 230 and 300 containing the leucine repeat sequence was essential for subunit binding, and much of the evidence indicating the involvement of other regions in dimerization had not yet been published. However, the results presented in this paper show that this region is not directly responsible for dimerization because it was found that the mutations only partially affected subunit interactions and did not completely inhibit them. Dimerization of the subunits was not prohibited by a mutation in the leucine repeat region because one or more of the other regions described above is probably also involved in subunit binding. Also, the leucine repeat region may be more important for intramolecular hydrophobic interactions which maintain the tertiary structure of the molecule and enable the subunits to associate properly so that subunit binding in other regions can take place. The data in this study suggests that the leucine repeat sequence is important for hydrophobic interactions; however, it is probably not the primary subunit binding region.

Another interesting question that this research raises is why the L289P mutation had a greater effect on subunit interactions than the L289R mutation? In order to decide which amino acid changes to make, a computer program was used called MacVector 3.5 which draws profiles of proteins based on the amino acid sequence of the protein and the chemical characteristics of each residue (78). For example, a hydrophilicity profile is determined by assigning each amino acid a "hydropathy" value based on the Kyte and Doolittle hydropathy scale (79) and using this value to determine the average hydrophilicity per residue for the protein (78). The hydrophilicity profile of wild type RT and the different L289 mutants is shown in Figure 50 and the site of the changed amino acid is circled in each case. As can be seen in this Figure, mutants L289R and L289P are different
Figure 50. Hydrophilicity profile drawn by MacVector computer program based on amino acid sequence characteristics. The RT sequence from amino acid 269 to 311 is shown for wild type RT (Panel A), mutant L289R (Panel B), and mutant L289P (Panel C). Amino acid 289 is circled showing where the site-specific mutation was made. Positive values are hydrophilic and negative values are hydrophobic.
Hydropathicity

A. Wild Type

B. L289R

C. L289P
from wild type RT but similar to each other. The L289R mutation has a slightly greater effect on the hydrophilicity profile than the L289P mutation. If hydrophobic interaction was the most important factor in the subunit interaction, then one might expect the L289R mutation to have a greater effect on dimerization than the L289P mutation which would be the opposite of what was actually observed. Surface probability is calculated by the MacVector program based on studies by Janin and coworkers (80) which assign each amino acid a percent exposed or percent buried value. These values were used by Emini et al. (81) to calculate a fractional surface probability for each amino acid, which MacVector uses to determine a running average of the fractional surface probability along the length of the protein (78). Figure 51 shows the surface probability calculations for wild type RT and mutants L289R and L289P. The results are similar to the results for hydrophilicity which is not surprising since surface probability takes into consideration the hydrophilicity or hydrophobicity of amino acids. As can be seen in Figure 51, the two mutants have similar profiles to each other but again are different from the wild type RT, and the L289R mutant shows a slightly greater difference than L289P. When the computer was asked to predict features of secondary structure some different results were obtained. Secondary structure predictions are carried out using a combination of two methods, the Chou-Fasman method (82) and the Robson-Garnier method (83), which are each graphed separately followed by a graph of consenting results from both methods. The Chou-Fasman method assigns each amino acid to one of four classes: helix formers, helix breakers, sheet formers, or sheet breakers, based on statistics of known x-ray crystal studies. These classifications are used to locate regions of helix or sheet forming residues which are assigned weights in order to calculate their significance (82). Predictions using this method may vary depending on how different programmers use weight calculations (78). The Robson-Garnier method is more clearly defined and should not vary among different program applications (83, 78). MacVector's application of this method determines the tendency for a given residue to be a part of either an alpha helix, a beta sheet, or a turn. Figure 52 shows the results of the
Figure 51. Surface probability profile drawn by MacVector computer program based on amino acid sequence characteristics. The RT sequence from amino acid 269 to 311 is shown for wild type RT (Panel A), mutant L289R (Panel B), and mutant L289P (Panel C). Amino acid 289 is circled showing where the site-specific mutation was made. Values greater than 0.50 represent amino acids that are more likely to be at the surface of the protein and values less than 0.50 are amino acids that are more likely to be buried.
Surface Probability

A. Wild Type

B. L289R

C. L289P
Figure 52. Secondary structure profile drawn by MacVector computer program based on amino acid sequence characteristics. The RT sequence from amino acid 269 to 311 is shown for wild type RT (Panel A), mutant L289R (Panel B), and mutant L289P (Panel C). Amino acid 289 is circled showing where the site-specific mutation was made.

CF = Chou-Fasman predictions for helix, sheet, or turns
RG = Robson-Garnier predictions for helix, sheet, or turns
CFRG = consensus predictions for helix, sheet, or turns
Secondary Structure

A. Wild Type

B. L289R

C. L289P

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secondary structure profiles for wild type RT and the two mutants. As can be seen in this figure, the structure of an L289R mutant was predicted to be similar to that of wild type RT, whereas the structure of the L289P mutant was predicted to be significantly different. It is possible that the proline residue, an amino acid which is usually found in the bends of large folded proteins, changed the secondary structure enough to hinder association of subunits and enhance dissociation of subunits in acetonitrile. Taken together, the predicted effects of the mutations help explain the observed results. Both L289R and L289P mutants differed from wild type RT in their hydrophilicity profile, but L289P also differed in secondary structure effects. Consequently, both mutants could have affected subunit interactions by disrupting the hydrophobic interface, but mutant L289P had an additional effect on secondary structure by inserting a kink in the amino acid chain which made it more difficult for the subunits to come together.

How do the results of this research compare with the research of Goel and coworkers (40) who also mutated leucine 289 of RT? They changed the leucine to a lysine in both the p66 subunit and the p51 subunit. These researchers used gel filtration, ultracentrifugation, circular dichroism analysis, and RT activity assays to analyze the effects of the mutations on dimerization. They found that the L289K mutation in the p51 subunit did not affect dimerization or RT activity but that the L289K mutation in the p66 subunit greatly reduced RT activity and dimerization. The apparent molecular weight as judged by SDS-PAGE was the same for wild type and mutant forms of RT. However, from circular dichroism spectroscopy they concluded that mutants L289K-p66 and L289K-p51 were structurally similar but not identical to their wild type homologues and that L289K-p66 showed a greater difference from wild type p66 than L289K-p51 from wild type p51. It is interesting, although not surprising, that the mutant which showed the greatest difference in structure from the wild type species was also the mutant which had the greatest effect on dimerization. Since our lab does not have the capability of circular dichroism spectroscopy, the results of the Goel study could not be directly compared to
ours on the basis of this technique. However, it would be expected that our L289R-p66 mutant would not show as great a difference in structure from wild type p66 as Goel observed for their L289K-p66 mutant since the activity of L289R-p66 was not as different from wild type p66 as Goel observed for L289K-p66. Based on SDS-PAGE analysis and analytical gel filtration, both of our p66 mutants migrated the same distance and eluted at the same time as wild type p66, indicating that not only the molecular size was the same, but also the three dimensional shape of the molecule was very similar. Molecules of the same size but different shape will migrate at different rates through a molecular sieve, for example, a globular molecule will run faster than a linear molecule of the same size. Using analytical gel filtration, the Goel study looked at dimerization of L289K-p51 with wild type p66 and with L289K-p66, as well as L289K-p51 by itself. They also looked at L289K-p66 with wild type p51 and they found that all of these combinations produced only monomer species except wild type p66 with L289K-p51, demonstrating that mutant L289K-p66 was not able to dimerize. These researchers did not use analytical gel filtration to study L289K-p66 homodimerization. However, they did use analytical ultracentrifugation to look at L289K-p66, and their results suggested that the polypeptide was a homogeneous monomer (40). For the research presented in this paper, L289R-p66 and L289P-p66 were analyzed using analytical gel filtration and intrinsic fluorescence studies for homodimerization only. Each mutant showed a decrease in the ability to dimerize with itself as compared to wild type p66, however they did dimerize. There are many possible reasons for the difference between the results presented here and the results observed in the Goel study. First of all, their p51 RT construct is 15 amino acids shorter than our p51, which terminates at the same amino acid residue as that generated by cleavage by HIV protease in vivo (11). Another difference is that their activity assay is carried out at 25°C for 10 minutes, while ours is at 37°C for 30 minutes. Their assay conditions may be suitable for wild type RT but not for the mutant RT. Also, the activity of the mutants was expressed as a fraction of wild type RT activity instead of specific activity as in our results.
Another difference is that they used a different buffer for analytical gel filtration and samples were incubated at 30°C for 2 hours before injecting them on the column. RT is very sensitive to changes in pH, temperature, ionic strength, and enzyme concentration. Goel and coworkers conclude that the leucine repeat sequence in the p66 subunit only is directly involved in and essential for subunit binding and enzyme activity of HIV-1 RT. Our evidence shows that the leucine repeat sequence is not essential for subunit binding and, although it is important for maintaining enzyme structure and activity, it would not be a good target for designing anti-HIV therapy. Certainly more research needs to be done in order to understand subunit binding, and to design a method of inhibiting it which would in turn inhibit enzyme activity. In the case of our mutants, more studies need to be done using the wild type p51 subunit to determine the effect of the p66 mutation on heterodimerization. Also, it would be useful to create the same mutations in the p51 subunit to determine if the role of this residue in this subunit is different than it is in the p66 subunit. Since this research implies that other regions of RT are more important for subunit binding it would be desirable to try mutations in these other regions. It may be that subunit binding involves several residues so that a single amino acid change is not enough to characterize such a complex protein-protein interaction. More studies need to be done on the effect of pH and ionic strength on dimerization. Since dimerization is essential for enzyme activity, this is still an important area for research to combat AIDS.
REFERENCES


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