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Site-Directed Mutagenesis of the Putative Processing Site of Recombinant Human Immunodeficiency Virus Reverse Transcriptase

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SITE-DIRECTED MUTAGENESIS OF THE PUTATIVE PROCESSING SITE OF RECOMBINANT HUMAN IMMUNODEFICIENCY VIRUS REVERSE TRANSCRIPTASE

by

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The human immunodeficiency virus (HIV) is the etiological agent of the acquired immune deficiency syndrome (AIDS). This retrovirus, encodes a RNA dependent DNA polymerase, reverse transcriptase (RT), which is essential to virus replication. Biochemical analysis indicated that RT is composed of 2 polypeptides of 66,000 and 51,000 daltons (p66 and p51) which combine to form a heterodimer. This heterodimer is thought to arise from the post-translational processing where p66 is made and either autocatalytic or proteolytic cleavage events result in a heterogeneous p66/p51. Site-directed mutagenesis was used at the proposed processing site to probe HIV-RT processing.

Three prokaryotic expression vectors each carrying a mutated form of RT were constructed. These mutants were induced and the product evaluated for RT enzymatic activity and the forms of RT protein visualized on SDS polyacrylamide gels and western blots. Results suggest that the mutations were at or near the RT processing site.

TABLE OF CONTENTS

Table of Contents--Continued

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Acquired immune deficiency syndrome (AIDS), an infectious disease affecting the human population, was first reported in 1981 (Centers for Disease Control Task Force, 1982). The clinical abnormalities associated with AIDS in humans are the result of functional abnormalities of the immune system (Laurence, 1987). Several studies by various research groups led to the identification of a retrovirus, named the Human Immunodefiency Virus (HIV), to be the etiological agent of AIDS (Gallo & Montagnier, 1987).

Understanding of the life cycle of this newly identified virus has provided information for the development of therapeutic strategies against HIV. HIV's ability to successfully integrate into a host cells genome depends on the virally encoded reverse transcriptase (RNA dependent DNA polymerase; Varmus, 1987). Since no cellular homolog to reverse transcriptase (RT) has been identified, it is a good therapeutic target.

Biochemical analysis of recombinant RT as well as RT isolated from virions indicated that RT is composed of 2 polypeptides of 66 and 51 KDa which combine to form a heterodimer (Chandra, Gerber, & Chandra, 1986; Lightfoote, et al., 1986). This heterodimer is thought to arise from the post-translational processing where p66 is made and either autocatalytic or proteolytic cleavage events result in a heterogeneous p66/p51 and smaller forms of the enzyme (Deibel, McQuade, Brunner, & Tarpley, 1989). Work by Deibel (1988) has suggested a small region of the p66 protein where processing may occur.

The strategy of this project was to use site-directed mutagenesis to introduce conservative amino acid changes into the proposed processing site of a recombinant HIV-RT to probe p66 processing. One goal was to obtain information supporting the importance of these amino acids in p66 processing while a long range goal was to block processing and obtain stable p66 which would be suitable for structural analysis.

CHAPTER II

REVIEW OF LITERATURE

History of AIDS

Acquired immune deficiency syndrome (AIDS) is an infectious disease affecting the human population. Heyward and Curran (1988) reviewed the epidemiology that led to the discovery of AIDS. The first indication that a new disease had arose was the increase in a rare cancer (Kaposi's sarcoma) among young white homosexuals. Michael S. Gottlieb of the University of California at Los Angeles School of Medicine, Frederick P. Siegal of the Mount Sinai Medical Center and Henry Masur of New York Hospital first reported this new syndrome in 1981. In addition, opportunistic infections, mainly pneumonia caused by Pneumocystis carinii, and a depletion of T4 cells were associated with AIDS. This infectious form of immune deficiency which leads to death was quickly found to be spreading among users of intravenous drugs, recipients of frequent blood transfusions and Haitians.

At the Cold Spring Harbor Workshop on AIDS in February 1983, Gallo proposed that AIDS was probably caused by a retrovirus, since the pattern was similar to that found with HTLV-I, a retrovirus associated with leukemia (Gallo & Montagnier, 1987). Several research groups took up the search, utilizing known methods for detecting retroviruses. In 1983 Luc Montagnier and coworkers at the Pasteur Institute published the first report of a new retrovirus (Barre-Sinoussi, et al., 1983) which was quickly followed by two publications by Robert C. Gallo and colleagues (Gelmann, et al., 1983; Gallo, et al., 1983), who had also isolated this retrovirus (although, later they were found to be different

strains). Therefore, in just two years the epidemic was clarified and a previously unknown virus, named the human immunodeficiency virus (HIV) was isolated and shown to cause the disease.

Since the early discovery of HIV in homosexual men, this disease has also become a leading cause of death in the United States among people with hemophilia and users of intravenous drugs (Heyward & Curran, 1988). The virus is primarily spread through sexual contact as well as through traces of blood in needles and from mother to infant. On September 23, 1989, the Center for Disease Control reported the cumulative 1989 total cases of AIDS in adults and children in the United States to be 25,555 (Morbidity and Mortality Weekly Report, 1989). The Public Health Service reports an estimate of between one and 1.5 million people in the United States to be infected with HIV. Whereas, the World Health Organization suggests that at least five million people worldwide are infected by the AIDS virus and a million new cases of AIDS are likely within the next five years (Mann, Chin, Piot, & Quinn, 1988).

The AIDS virus is believed to have originated in Africa (Essex & Kanki, 1988). HIV has relatives in monkeys; simian immunodeficiency virus (SIV) was isolated in both captive Asian macaque (Kanki, et al., 1985) and wild green monkeys in Africa (Kanki, Alroy, & Essex, 1985). The Asian macaque shows clinical signs of the disease while the African green monkey does not. Genetic studies have shown approximately 50 percent homology between the nucleotide sequence of HIV and SIV (Essex & Kanki, 1988). Later, a second retrovirus (HIV-2), also thought to cause AIDS, was found in humans in West Africa (Clavel, et al., 1986). This virus is much more closely related to SIV (Guyader, et al., 1987). Therefore, one possible hypothesis concerning the origin of HIV is that SIV entered humans and through a series of mutations became HIV which causes the destructive pathology of AIDS (Essex & Kanki, 1988).

Anti-AIDS Therapies

The clinical symptoms associated with HIV infection in humans are the result of functional abnormalities of the immune system. Once the virus is inside the body its target consists of cells bearing the T4 molecule on their outer membrane. T4 lymphocytes as well as monocytes and macrophages are in this category and these cells are important for proper communication with other cells in the immune system. The breakdown of the immune system responses which depend on T4 cells include: (a) antibody production, (b) cytotoxic T-cell activity, (c) T-cell proliferation induced by interleukin-2, and (d) macrophage stimulation by gamma-interferon (Laurence, 1987). Several immunologic studies (Edelman & Zolla-Pazner, 1989) suggest that these immune abnormalities become more profound as each comes into play, eventually leading to a break down of immune defenses to a point where opportunistic infections and malignancies can develop.

Yarchoan, Mitsuya, & Broder (1988) reviewed strategies for the development of therapy against AIDS. These include classes of drugs which inhibit viral binding, arrest viral protein synthesis, inhibit enzymes that trim sugar groups from viral proteins, reduce viral budding, induce synthesis of interferon, and inhibit viral reverse- transcriptase activity. The target which has received the most attention is the activity of reverse transcriptase because it is unique to retroviruses. Presently the only FDA approved drug therapy is a reverse transcriptase inhibitor, 3'-azido-2',3'-dideoxythymidine (AZT). AZT is a dideoxynucleoside which functions through its resemblance to the nucleoside thymidine, where in the cell AZT is converted to AZT triphosphate and is substituted for thymidine triphosphate in the synthesis of viral DNA. This results in competitive inhibition and chain termination (Mitsuya, et al., 1985). In spite of AZT's effectiveness, the drug is toxic, particularly to bone marrow, so a need for other less toxic reverse

transcriptase inhibitors remains (Yarchoan, et al., 1988). Presently, the National Institute of Health sponsors a broad AIDS drug development program (Fox, 1989).

Human Immunodeficiency Virus Life Cycle

A knowledge of the HIV life cycle is required for the design of drug therapies aimed at specific stages of viral replication. Haseltine and Wong-Staal (1988) summarized the molecular biology of the AIDS virus. The cycle begins when an HIV particle binds to the receptor of a cell and the viral membrane fuses with the cell membrane. This fusion allows its core, which includes two identical strands of RNA as well as structural proteins and enzymes that carry out later steps in the life cycle, to enter the cell. Once inside the cell the DNA polymerase activity of RT results in a single-stranded DNA copy of the viral RNA while the ribonuclease activity of RT destroys the original RNA. Then the polymerase synthesizes a second DNA copy, using the first one as template. This double-stranded DNA migrates to the host cell nucleus where another enzyme, called integrase, randomly incorporates the viral DNA into the host genome. The viral DNA, called a provirus, is duplicated with the host cell's DNA whenever the cell divides.

Later events of the viral life cycle involve production of new virus paticles beginning when the nucleotide sequences of the viral long terminal repeats direct enzymes of the host cell to copy the DNA of the integrated virus into RNA. The virions are assembled from multiple copies of two different protein molecules. The more abundant protein is the precursor of the protein shell that will enclose the RNA and enzymes in the maturing virions. The other molecule contains the structural components and segments which will become the viral enzymes. Multiple copies of the two proteins and the two copies of viral RNA migrate and attach to the cell membrane forming a

spherical bulge. One of the viral enzymes, a protease, cuts free from the precursor protein and cleaves other enzymes forming reverse transcriptase and integrase and a core is formed around the RNA and enzymes. Assembled and transported independently of the core proteins is the envelope protein (gp160) which surrounds the newly assembled virion; this envelope is studded with glycoprotein (gp120) and provides infectivity to the new virion. The mature virion then pinches off from the host cell membrane to become free virus. Therefore, the genes which encode for these replicative events include gag (core proteins), pol (enzymes), and env (envelope protein).

In addition to the gag, pol, and env genes, the HIV genome includes at least six other genes which act to regulate the production of viral proteins. The tat gene or trans-activator is responsible for initiation of replication while a second gene rev has differential effects. It enables the integrated virus to produce selectively either regulatory proteins or virion components as well as being important in transporting HIV RNA from the nucleus to the cytoplasm. A third gene called nef (negative-regulatory factor), may be responsible for the ability of HIV to turn off its own growth and become latent. Two newly identified genes, vpr and vpu, may also be involved in regulation of viral replication. The gene vif (virion infectivity factor) encodes a small protein that somehow enhances the ability of the virus that has budded from one cell to infect another.

Reverse Transcriptase

RT is encoded by the HIV pol gene (Figure 1). This gene expresses several proteins (Loeb, Hutchison, Edgell, Farmerie, & Swanstrom, 1989); an 11 KDa protein, thought to be a protease, RT (66 and 51 KDa forms), and a protein of 34 KDa which is the viral integration protein. RT subunits p66 and p51 share a common amino terminus (Chandra,

et al., 1986; Lightfoote et al., 1986) while the ribonuclease H activity of RT is encoded at the carboxyl terminus (Johnson, McClure, Feng, Gray, & Doolittle, 1986). The 66 and 51 KDa forms of RT have been shown to occur in approximately equal proportions by immunoaffinity purification (Starnes, Gao, Ting, & Cheng, 1988). Production of recombinant RT in bacterial cells (Barr, Power, Lee-Ng, Gibson, & Luciw, 1987; Farmerie et al., 1987; Hansen, Schulze, & Moelling, 1987; Hizi, McGill, & Hughes, 1988; Larder, Purifoy, Powell, & Darby, 1987; Larder, et al., 1988; Le Grice, Beuck, & Mous, 1987; Mous, Heimer, & Le Grice, 1988) has allowed for further characterization of the p66/p51 RT template specificity (Cheng, Dutschman, Bastow, Sarngadharan, & Ting, 1987; Hoffman, Danapour, & Levy, 1985), and nucleotide sequence (Muesing, Smith, Cabradilla, Lasky, & Capon, 1985; Ratner, et al., 1985; Sanchez- Pescador et al., 1985; Wain-Hobson, Sonigo, Danos, Cole, & Alizon, 1985).

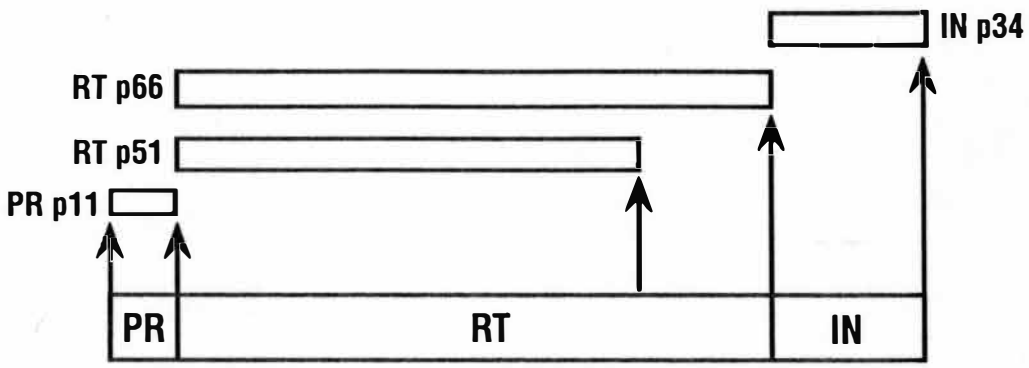

Figure 1. HIV pol gene and expressed products. Protease (PR) produces an 11 KDa protein, reverse transcriptase (RT) produces two forms (66 and 51 KDa), and integrase (IN) produces a 34 KDa protein.

Source: Loeb, D. D., Hutchison III, C. A., Edgell, M. H., Farmerie, W. G., & Swanstrom R. (1989). Mutational analysis of human immunodeficiency virus type 1 protease suggests functional homology with aspartic proteinases. Journal of Virology, 63(1), 111-121.

More biochemical and structure/function relationship studies as well as knowledge of the three-dimensional structure of RT are needed in order to design therapeutic strategies which would be successful against HIV RT. Since no cellular homolog of viral RT has been discovered, it is a good target for drug therapies which interfere with the retroviral life cycle (Varmus, 1987). Presently, nucleoside triphosphate analogs, particularily zidovudine (AZT) have shown some clinical success (Cheng et al., 1987; Furman et al., 1986; Mitsuya et al., 1985), confirming the idea that RT is a good therapeutic target.

The two polypeptides (p66 and p51) of RT exist under native conditions as a heterodimer, which arises from p66 by autocatalytic and/or proteolytic processing (Deibel, et al., 1989). Heterodimer formation is also associated with the formation of multiple minor p66/p51 derivatives (Barr et al., 1987; Farmerie et al., 1987; Hansen et al., 1987; Hansen, Schulze, Mellert, & Moelling, 1988; Le Grice, Zehnle, & Mous, 1988; Lowe et al., 1988; Mous et al., 1988; Tisdale et al., 1988). In order to utilize three dimensional crystallography to aid in the design of specific inhibitors of HIV-RT, a homogeneous preparation of p66 (free of p66/p51 microheterogeneity) is needed (Lowe et al., 1988). Work by Deibel (1988) has suggested a small region of p66 where processing may occur. Positive identification of the RT processing site would greatly enhance understanding of this unique enzyme and how it functions.

CHAPTER III

MATERIALS AND METHODS

Research Project Strategy

Figure 2 depicts the strategy to be used for this project. A RT restriction fragment containing the proposed processing site will be removed from a genetically engineered plasmid containing the portion of the HIV pol gene which encodes RT. This fragment will be cloned into the M13mp18 phage vector which is suitable for site-directed mutagenesis.

Three synthetic oligonucleotides will be designed which, by a single base change, would result in different amino acid substitutions at or near the proposed processing site; this site consists of the amino acids glutamine (Q), leucine (L), glutamic acid (E), lysine (K), and a second glutamine. Oligonucleotide Smp3 (Figure 2) will be designed to change the first glutamic acid (E) to glutamine (Q), Smp4 to change the second glutamic acid to glutamine, while Smp5 would change both glutamic acids to glutamine. These substitutions would change a negatively charged protein into a neutral one.

The mutant oligonucleotide will be purified, 5'-phosphorylated, and then annealed to single-stranded template (prepared from M13mp18-RT phage). The oligonucleotide will then be extended with DNA polymerase I (large fragment) from E. coli and ligated with T4 DNA ligase to generate a mutant heteroduplex. During this reaction the dCTP of the mutant strand will be replaced by dCTPalphaS (a thionucleotide), thereby, allowing removal of the non-mutant strand which will be nicked by the restriction enzyme Nci 1 and digested by exonuclease III from E. coli.

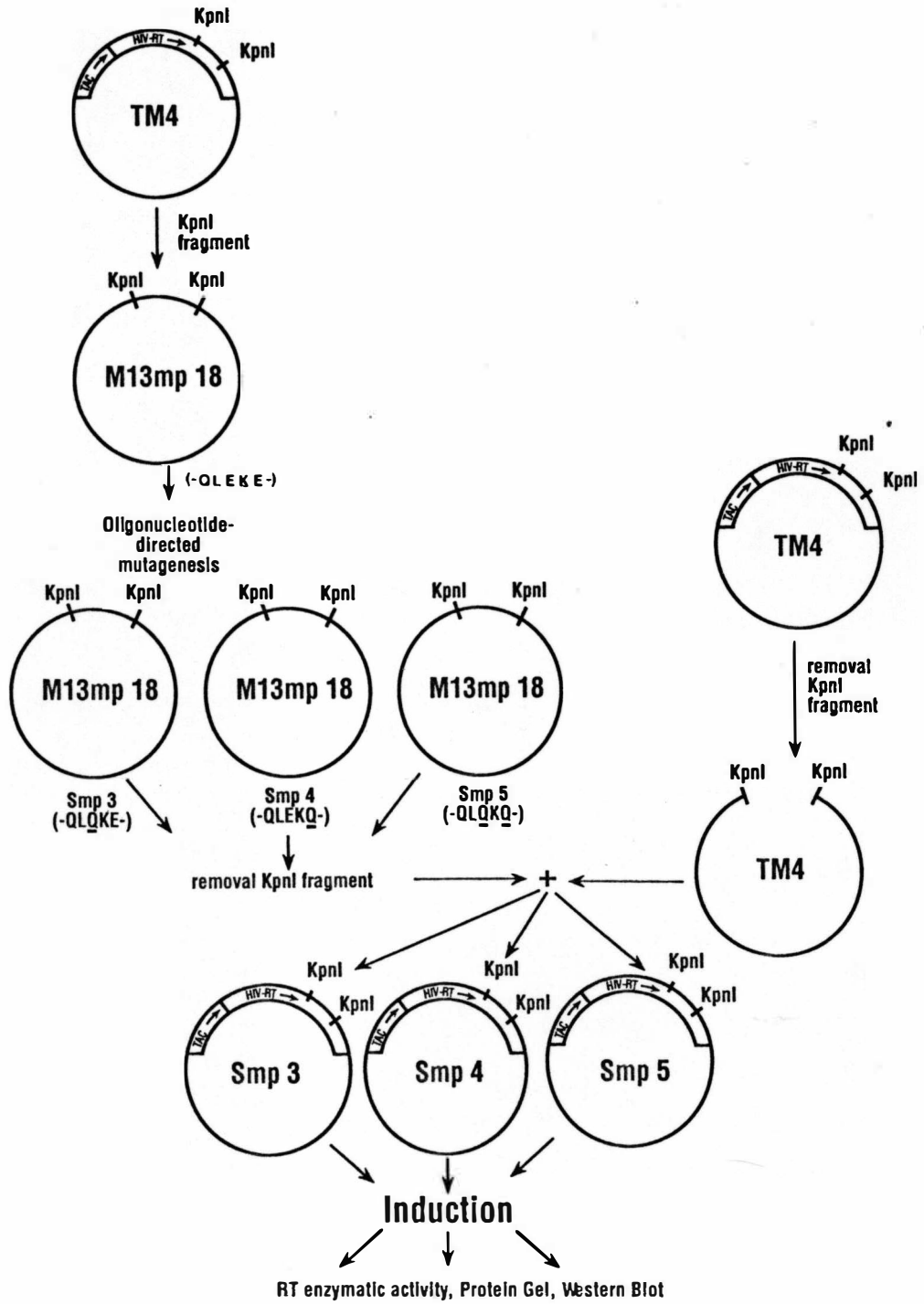

Figure 2. Research strategy. Three expression vectors were engineered which produce a mutated form of the HIV reverse transcriptase. Amino acids: Q is glutamine, L is leucine, E is glutamic acid, and K is lysine. Changed amino acids are underlined.

Selective removal of the non-mutant strand is made possible by the incorporation of the thionucleotide into the mutant strand during synthesis since certain restriction enzymes cannot cleave phosphorothioate DNA (Taylor, Schmidt, Cosstick, Okruszek, & Eckstein, 1985). The mutant strand will then used as a template for reconstruction of the double-stranded molecule. The double-stranded molecule will then be transformed into the host cell (E. coli TG1) and recombinant phage extracts will be prepared.

Selection of the mutants will be accomplished by dot blot hybridization with the appropriate oligonucleotide probe labeled at the 5'-end with ^{32}P and dideoxy DNA sequencing of the mutant region. The selected mutants will be grown, and a restriction fragment containing the mutation will be isolated and reinserted into the original plasmid vector restoring the complete recombinant RT nucleotide sequence. The resulting three mutant RT genes are predicted to encode the recombinant HIV-RT with only one (Smp3 and Smp4) or two (Smp5) amino acid changes. Transformation into E. coli JM109 will prepare the mutants for analysis.

The expression vector to be used contains the strong B-isopropyl- beta-D-thiogalactoside inducible TAC promoter (De Boer, Comstock, & Vasser, 1983) located upstream from HIV-RT (Figure 2). The recombinants will be induced to produce the mutant RT. The bacterial extracts will be examined for enzymatic activity by DNA synthesis in vitro from an RNA template/DNA primer. The molecular clones having elevated RT activity will be selected for further analysis. These mutants will be grown, induced and prepared for electrophoresis on SDS- polyacrylamide gels. One gel will be stained for visualization of the proteins while an identical gel will be electroblotted onto nitrocellulose and subjected to a western blot using pooled AIDS patient antisera known to contain anti-RT antibodies to reveal the target protein bands. Using this strategy, the processing of HIV-RT to form the p66/p51 heterodimer will be studied.

Preparation of DNA for Mutagenesis

The components of reagents and media used are located in Appendix A while information on suppliers is located in Appendix B. The original plasmid (TM4) with the HIV-RT sequence was received from Thomas McQuade at the Upjohn Company. Initially, this plasmid was digested with the restriction enzyme Kpn I (New England Biolabs) to confirm the presence of the expected 328 base pair (bp) fragment corresponding to the region of the HIV-RT sequence containing the proposed processing site. The digest was electrophoresed on a 1% Sea Kem GTG (FMC BioProducts) agarose gel prepared in TBE buffer. The gel was then stained in a 2.5 microgram/ml ethidium bromide solution for ten minutes and photographed under an ultra violet light source. Two fragments were seen: A 328 bp fragment and a 6 kb fragment confirming the identity of TM4. Next, 20 micrograms of TM4 were similarly digested and the 328 bp fragment recovered and purified using a NACS column (Bethesda Research Laboratories) with 0.2M NaCl for loading and 1.0M NaCl for eluting the DNA fragment. The DNA was ethanol precipitated and resuspended in TE buffer.

The vector M13mp18 (New England Biolabs) was selected as the recipient for the RT fragment since it is capable of producing single- stranded phage DNA needed for *in vitro* mutagenesis. Two micrograms were digested with Kpn I as above, extracted with phenol and chloroform, ethanol precipitated and resuspended in TE. The recovered vector was electrophoresed on a 1% agarose gel to confirm linearization.

Six tenths microgram of the linearized M13mp18 and 0.08 microgram of the RT fragment were ligated at 16°C in 1mM ATP, ligase buffer and T4 DNA ligase (1 Unit, New England Biolabs). The ligation mix was transformed into competent *E. coli* TG1 host cells (Amersham).

TG1 cells were received freeze dried, resuspended in 2X TY medium and plated onto 2X TY agar. Individual colonies were then maintained on glucose/minimal medium plates to select for those cells carrying the F-plasmid.

To prepare competent cells, an overnight culture of TG1 cells from a glucose/minimal medium plate was used to inoculate fresh 2X TY medium. The cells were grown to optical density (OD) at A_{550} of 0.3, spun at 478xg for 5 minutes, resuspended in cold 50mM $CaCl_2$, placed on ice for 20 minutes, respun at 478xg for 5 minutes and finally resuspended in cold 50mM $CaCl_2$. Transformation involved mixing an aliquot of these cells with the ligation mix, cooling on ice for 40 minutes, heat shocking for 45 seconds at 42°C, cooling for 5 minutes and then plating.

Plating out was accomplished by mixing 100mM B-isopropyl-beta-D-thiogalactoside (IPTG), 2% X-gal and 200 microliters/plate of log phase TG1 cells with DNA aliquots of 3, 30, and 300 ng of the transformed TG1 cells, adding this to molten H top agar and pouring onto a prewarmed H plate. After overnight incubation at 37°C phage plaques from transformed cells carrying the RT insert were selected by their clear appearance (transformed TG1 cells with M13mp18 without the insert produced blue plaques). Eight clear plaques were purified by replating on IPTG/X-gal indicator plates.

Phage cultures of the M13mp18/RT recombinants were prepared as follows. An overnight culture of TG1 cells from a glucose/minimal medium plate was prepared. The following morning, one drop of the overnight culture was added to 20 ml of 2X TY medium and grown at 37°C for 3 hours. A recombinant plaque was then used to inoculate 0.3 ml of the 3 hour TG1 culture in 1 ml of 2X TY medium. After incubation for 4 hours, the culture was spun at 5,800xg for 2 minutes. The supernatant, containing the phage, was stored at 4°C.

To release M13mp18/RT DNA, the cells were spun down at 5,800xg for two minutes and the pellet was resuspended in STET buffer with 1 mg/ml lysozyme and boiled for 45 seconds; then spun at 11,600xg for 10 minutes and the pellet removed. The supernatant was ethanol precipitated and the recovered DNA resuspended in TE buffer. Restriction enzymes were selected which would cut fragments of appropriate sizes to determine the orientation of the RT fragment and the presence of multiple inserts. Based on the results of these restriction digestions, a M13mp18/RT clone was selected.

The selected M13mp18/RT phage was plaque purified by plating dilutions onto IPTG/X-gal indicator plates. Single-stranded template DNA was prepared by inoculation of a TG1 cell culture with a plaque, incubating for 5 hours at 37^0C, and collecting the supernatant. Twenty percent (w/v) polyethylene glycol 6000 in 2.5M NaCl was mixed with the supernatant and the phage recovered, phenol extracted, and finally ethanol precipitated. The purity of the single-stranded template was verified in a 1% agarose gel with 0.5 mg/ml EtBr.

Site-Directed Mutagenesis

Three synthetic oligonucleotides, designed to contain a single base mismatch, were synthesized by Biopolymer Chemistry at the Upjohn Company. The crude extracts were purified on a 20% polyacrylamide (Bio-Rad) gel, desalted using a Waters Associates SEP-PAC C18 cartridge, dried in a Speed-Vac (Savant) and the oligos resuspended in TE buffer. Each oligonucleotide was 5' phosphorylated in the presence of kinase buffer A and T4 kinase (10 Units, New England Biolabs).

The Amersham Oligonucleotide-directed in vitro Mutagenesis System was used to create the mutants (Figure 2). Annealing took place by mixing appropriate molar quantities of each phosphorylated oligonucleotide with the single-stranded

M13mp18/RT template. This mixture was incubated at 70°C for 3 minutes and then at 37°C for 30 minutes. Synthesis and ligation of the mutant DNA strand followed by adding dNTPs, Klenow fragment, and T4 DNA ligase. This mixture was incubated overnight at 16°C. During this reaction the dCTP of the mutant strand was replaced by dCTPalphaS, a thionucleotide. Single- stranded non-mutant DNA was removed by filtering and the mutant DNA was ethanol precipitated. Nicking of the non-mutant strand of the heteroduplex was accomplished by adding the restriction enzyme NCI 1 and incubating at 37°C for 90 minutes. NCI 1 only cuts at unprotected non-phosphorothioate sites on the non-mutant strand of the heteroduplex. The non-mutant strand was digested by exonuclease III at 37°C for 30 minutes and then the enzyme was inactivated at 70°C for 15 minutes. Resynthesis of double-stranded closed circular DNA in the form of homoduplex mutant molecules was completed in the presence of dNTPs, DNA polymerase I and T4 DNA ligase at 16°C for 3 hours. This final mutant form was transformed into TG1 cells and plated in the presence of IPTG/X-gal. A control reaction was run simultaneously using control template and oligonucleotides provided by Amersham.

The mutagenesis reaction was analyzed by electrophoresis of samples removed at each step of the procedure. Comparison of the control reaction and the mutagenesis reactions indicated that they occurred as expected.

Mutant Selection

Initially, selected mutant plaques were screened by a dot blot hybridization technique. First, a labelled probe was made by mixing 3 OD_{260} units of oligonucleotides per ml with 10 mCi/ml ^{32}P ATP (400 Ci/mmol, Amersham) in the presence of polynucleotide kinase (10 Units, New England Biolabs) and kinase buffer B, incubating at

37°C for 30 minutes and filtering. Hybridization took place by spotting mutant phage onto nitrocellulose filter (NF; BA85, 0.45 micrometer, Schleicher & Schuell), and baking at 80°C for 2 hours. Next, prehybridizing was in a solution of SSC, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, and 0.2% SDS for 1 hour at 67°C. The NF was washed in 6X SSC and the probe added. Hybridization was at 67°C for 30 minutes. Cooling to room temperature was allowed for over 30 minutes. The membrane was washed three times with 6X SSC at room temperature. X-ray film (Eastman Kodak Co.) was exposed to the NF in an exposure cassette. Several exposures were taken with ambiguous results, so the membrane was rewashed at 5°C below the calculated Td (4 x number of G,C bp plus 2 x number of A,T bp) and exposed again. This exposure allowed for the mutant phage which successfully hybridized to be selected for DNA sequencing.

The sequencing template was prepared by the following method. First, the RF (double-stranded) form of the M13mp18/RT mutants were grown as previously described. The recovered pellets were lysed by 2 mg/ml lysozyme in 1% glucose, 10mM EDTA, and 25mM Tris-HCl, pH 8.0 at 4°C for 30 minutes. Two-tenths molar NaOH and 1% SDS were added. After 5 minutes at 4°C, 3M NaOAc, pH 4.8 was added and incubation continued another 30 minutes. After spinning, the supernatant was recovered, ethanol was added and precipitation allowed to continue overnight. The next day, the pellet was recovered and resuspended in TE buffer, RNase A was added to 2 mg/ml and this mixture incubated for 10 minutes at room temperature. Phenol/chloroform extraction and ethanol precipitation followed. The recovered mutant template was resuspended in TE buffer.

The dideoxy sequencing chemistry reactions were carried out using the K/RT Sequencing System (Promega) following supplier instructions. A M13 reverse sequencing primer (New England Biolabs) was used which would allow for the mutant region of RT

to be sequenced. Avian Myeloblastosis Virus reverse transcriptase was the enzyme selected while ^{32}P dATP (400 Ci/mmol; Amersham) was used for radiolabelling.

A 0.4 mm, 8% polyacrylamide/7M urea sequencing gel was poured and assembled onto BRL-S2 apparatus (Bethesda Research Laboratories). The gel was prerun at 1700v for about 2 hours using 1X TBE as running buffer. The samples were loaded and the gel run at 1800v (45mA) with the temperature between 45 and 50°C. The gel was fixed in 10% acetic acid and 10% ethanol, washed with water, adhered to 3M Whatman paper, and dried under house vacuum for 1 hour, at 60°C. After exposure to X- ray film, the sequences were read. One of each of the desired mutants was selected for further analysis.

Preparation of Inducible Mutants

Pure double-stranded RF DNA was prepared for cloning from the selected (Smp3, Smp4, and Smp5) mutant phage stocks. Infected TG1 cells were grown as previously described, resuspended in cold 50mM Tris-HCl, pH 7.5 and 20 mg/ml lysozyme, incubated on ice for 10 minutes, following which 0.5M EDTA was added and incubation continuted for 10 more minutes. Triton X-100 was added to 0.8%, the mixture spun at 11,950xg for 45 minutes, and the supernatant stored overnight at 4°C. Next, RNase was added to 20 micrograms/ml, the mixture incubated at 37°C for 30 minutes, proteinase K added to 50 micrograms/ml, and incubation continued for another 30 minutes. The supernatant was collected and 5M NaCl and 1M MOPS, pH 7.0 added. The DNA was further purified by passing it through a QIAGEN-pack 100 column, eluting was by 1500mM NaCl, 50mM MOPS, and 15% ethanol (pH 7.5). The recovered DNA was ethanol precipitated and resuspended in TE buffer.

Each mutant RT segment was restriction digested with Kpn I to remove it from the M13mp18 vector, the fragment separated on a 1% agarose gel, and purified by a NACS column.

The plasmid TM4 was prepared to receive the mutated KpnI-KpnI RT fragment. Five micrograms of TM4 was restriction digested with Kpn I, separated on a 1% agarose gel, the linearized vector recovered, and purified through a NACS column. This procedure was repeated twice to ensure complete digestion.

Ligation of each mutant RT fragment and the TM4 vector restored the complete recombinant RT nucleotide sequence. These were transformed into JM109 cells. The resulting three mutant RT genes are predicted to encode the recombinant HIV-RT with only one (Smp3 and Smp4) or two (Smp5) amino acid changes due to the precise mutants engineered. Twenty-four transformants were selected from each ligation and screened to find the correct mutant.

The recombinant plasmids were induced to express the expected altered products by growing in Luria-Bertani medium containing 50 micrograms/ml ampicillin for 3 hours. Then IPTG was added to 1mM and the culture allowed to grow for another 2 hours. Pellets were collected and resuspended in TEN_{100}, and lysed with lysozyme. This culture was then spun (11,600xg) after the addition of NP-40 (Fluka Chemie). The soluble RT fraction was collected and evaluated for RT enzymatic activity.

RT enzymatic activity was evaluated by a modified assay reported by Hansen et al. (1987) which quantifies DNA synthesis <u>in vitro</u> from a RNA template/DNA primer. Two microliters of extract were incubated in a reaction mix that contained RT buffer, 10 micromole alpha ^{35}S dTTP (final specific activity 1 Ci/mM; Amersham), 10 micrograms/ml RNA template (poly rA), and 5 micrograms/ml DNA primer (oligo dT). The mixture was incubated for 10 minutes at 37°C and then spotted on paper three times. After drying,

the papers were washed 3 times each with 2X SSC and 95% ethanol for 5 minutes to remove unincorporated radioactive precursors. The papers were air dried, immersed in Aqueous Counting Scintillant (Amersham), and the ^{35}S incorporation measured by counting in a scintillation counter. Initially, this procedure was run using TM4 to determine the optimum reaction time for evaluation of enzymatic activity. Extracts which appeared partially to fully active as compared to the positive control of unmutated TM4 were characterized further.

Selected mutants were prepared for restriction digestion by the previously described plasmid mini-prep. Separate digestions with NSI I or EcoRI and Hind III allowed for determination of the clones with single copies of the insert in the proper orientation.

Analysis of Mutants

For further characterization of the selected mutants Smp3, Smp4, Smp5, and TM4, new RT extracts were prepared and collected at 0.25, 0.5, 1.0, 1.5, 2.0, and 24 hours after induction and enzymatic activity determined by the procedure described above.

In order to visualize the protein expressed by the RT mutants, samples were prepared for electrophoresis on SDS-polyacrylamide gels. The mutants were grown to OD_{600}, induced and harvested two hours later. Cell pellets were collected and resuspended in water, 2X sample prep buffer was added and the mixture boiled for 4 minutes, after cooling bromphenol blue dye was added to 5%. Three tenths of an OD_{600} unit were loaded onto each lane of two identical Integrated Separation Systems (Enprotech) mini gels (10-20% gradient) and electrophoresed at 30 mAmps. One gel was dyed and fixed in a solution of 0.25% Coomassie brillant blue, 45% methanol, and 10% glacial acetic acid, and destained in 45% methanol and 10% glacial acetic acid. The other gel was used for a western blot.

The proteins were transferred from the gel to NF in the presence of 25mM Tris-HCl, pH 7.4, 200mM glycine, and 20% methanol using a Hoefer Scientific transblotter. The NF was blocked overnight at 4°C by placing in 0.05% Tween 20 (Sigma) and 5% Carnation Instant Milk in Dulbecco's Phosphate Buffered Saline solution (Irvine Scientific). Next, the NF was exposed to the anti-RT antibodies (consisting of #444 pooled serum from AIDS patients, obtained from Dr. Lionel Resnick, University of Miami, FL.), NETG buffer, and 0.05% NP-40 for 2 hours at room temperature. The membrane was washed 5 times with 0.001% NETG, 0.5% Triton X-100 (Eastman Kodak Co.), and 0.1% SDS; one time with NETG, 0.5% Triton X-100, and 0.1% SDS for 15 minutes; 5 times with 0.001% NETG, 0.05% Triton X-100, and 0.1% SDS; and one time with blocking buffer. The membrane was then exposed to 1 part ^{125}I Protein A (79.8 microcurries/mmol; New England Nuclear) in 1000 parts NETG/0.05% NP-40 for 3 hours at room temperature. The membrane was again washed as above excluding the wash with blocking buffer and air dried. An autoradiograph was prepared. Additionally, the western blot procedure was completed for cell extracts collected at 0.25, 0.5, 1.0, 1.5, and 2.0 hours after induction.

CHAPTER IV

RESULTS

Construction of Mutants

Construction of three prokaryotic expression vectors was accomplished as described in Materials and Methods. An in vitro mutagenesis system was used to create single base changes in the region of the HIV reverse transcriptase nucleotide sequence believed to encode critical amino acids important in p66 processing. These nucleotide substitutions resulted in changes in the encoded amino acids. Table 1 depicts the amino acid sequence changes engineered.

Table 1

Changes of HIV Reverse Transcriptase Amino Acids 428-432

NATIVE TM4
glutamine - leucine - glutamic acid - lysine - glutamic acid
MUTANT Smp3
glutamine - leucine - glutamine - lysine - glutamic acid
MUTANT Smp4
glutamine - leucine - glutamic acid - lysine - glutamine
MUTANT Smp5
glutamine - leucine - glutamine - lysine - glutamine

Mutant Smp3 changed the first glutamic acid to glutamine, mutant Smp4 changed the second glutamic acid to glutamine, while mutant Smp5 changed both glutamic acids to glutamine. These changes replaced one or two negatively charged amino acids with an uncharged amino acid. The HIV-RT mutants were under the transcriptional control of the strong, inducible TAC promoter which after induction produced high levels of mutant reverse transcriptase.

Other investigators have also used a similar strategy to analyze HIV reverse transcriptase. Larder, Purifoy, Powell, & Darby (1987) used site-directed mutagenesis to study important functional regions of the protein while both Hizi et al. (1988) and Loeb et al. (1989) used deletion and insertion mutagenesis for similar analyses.

Reverse Transcriptase Enzymatic Activity

RT enzymatic activity was measured by incorporation of the DNA precursor dTTP into DNA from a RNA template. In Table 2 the results of dTTP incorporation in the presence of aliquots of E. coli extracts prepared at different times after protein induction are shown.

Extracts from JM109 without recombinant plasmids showed insignificant RT activity. JM109 carrying Smp3, Smp4 and the non-mutant TM4 showed increased RT activity with time up to 2 hours but diminished activity at the 24 hour time point. The mutant Smp5 showed about 10 times lower activity.

Table 2

Picomoles dTTP Incorporated in 10 Minutes
for 10 Microliters of Cell Extract

JM109 and Plasmids	Time (hour) after induction					
	0.25	0.5	1.0	1.5	2.0	24.0
Smp3	9.16 ± 0.52	10.77 ± 0.01	14.99 ± 0.35	18.08 ± 0.25	20.08 ± 0.14	0.24 ± 0.02
Smp4	5.04 ± 0.22	12.10 ± 0.18	12.89 ± 0.31	13.58 ± 0.14	17.10 ± 0.06	1.99 ± 0.11
Smp5	0.38 ± 0.02	0.64 ± 0.02	1.14 ± 0.02	1.20 ± 0.04	1.80 ± 0.03	2.24 ± 0.10
TM4	5.50 ± 0.24	9.49 ± 0.31	14.76 ± 0.04	16.80 ± 1.82	16.80 ± 0.12	3.73 ± 0.31
None	Not done	Not done	Not done	Not done	8.22×10^{-5} ± 0.10	5.41×10^{-5} ± 1.35

SDS Polyacrylamide Gel Electrophoresis

E. coli extracts from all samples were also prepared for analysis on SDS-polyacrylamide gels as described in Materials and Methods. Results showed a major protein was produced by TM4 of 66 KDa, the size expected for RT. This band was not seen in the extract of JM109. In the lanes carrying extracts from the mutant preparations, distinct bands differing from the JM109 bands were not seen. However, there were indications that Smp3 produced a faint 66 and a 51 KDa protein; Smp4 produced a 66 KDa protein; and Smp5 produced a 51 KDa protein (data not shown).

Western Blot Analysis

To confirm the results of SDS polyacryamide gel electrophoresis, a western blot was used to detect RT. The primary antibody used was pooled AIDS patient sera known to contain high levels of anti-RT antibodies. Identical extracts to those used above were subjected to this procedure as described in Materials and Methods. Figure 3 shows the results. As compared to TM4, mutant Smp3 had bands at the 66 and 51 KDa positions with the 51 KDa band being darker. Smp4 had a 66 KDa band while Smp5 had a 51 KDa band.

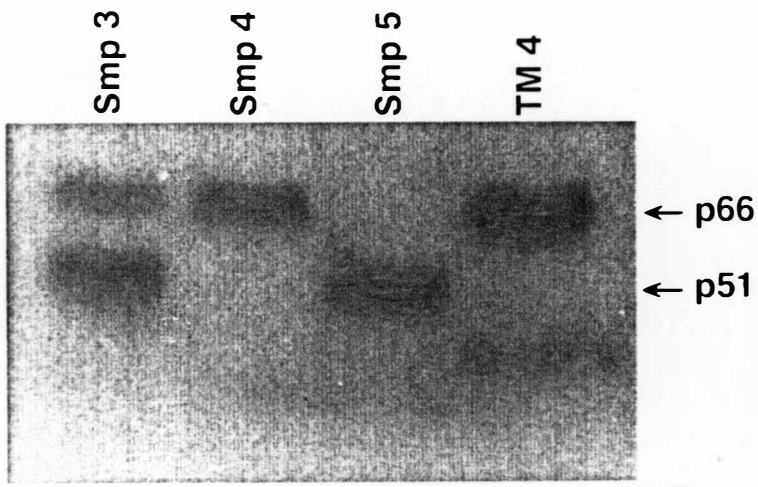

Figure 3. Autoradiograph from western blot on samples harvested two hours post-induction. Both forms of RT (p66 and p51 KDa proteins) were found as shown for the mutants Smp 3, 4, and 5 and native RT (TM4).

Extracts harvested at different times after induction were also subjected to a western blot and the results are shown in Figure 4. At time 0.25 hour, Smp3 produced bands at both the 66 and 51 KDa positions while Smp4 and TM4 showed bands at only the 66 KDa position. By 0.5 hour TM4 also had a 51 KDa polypeptide band visible

whereas Smp4 did not show this band until 1 hour after induction. Mutant Smp5 only produced a band at the 51 KDa position at all time points evaluated. Based on the results of these western blots, the appearance of the 51 KDa polypeptide occurred at different times post-induction.

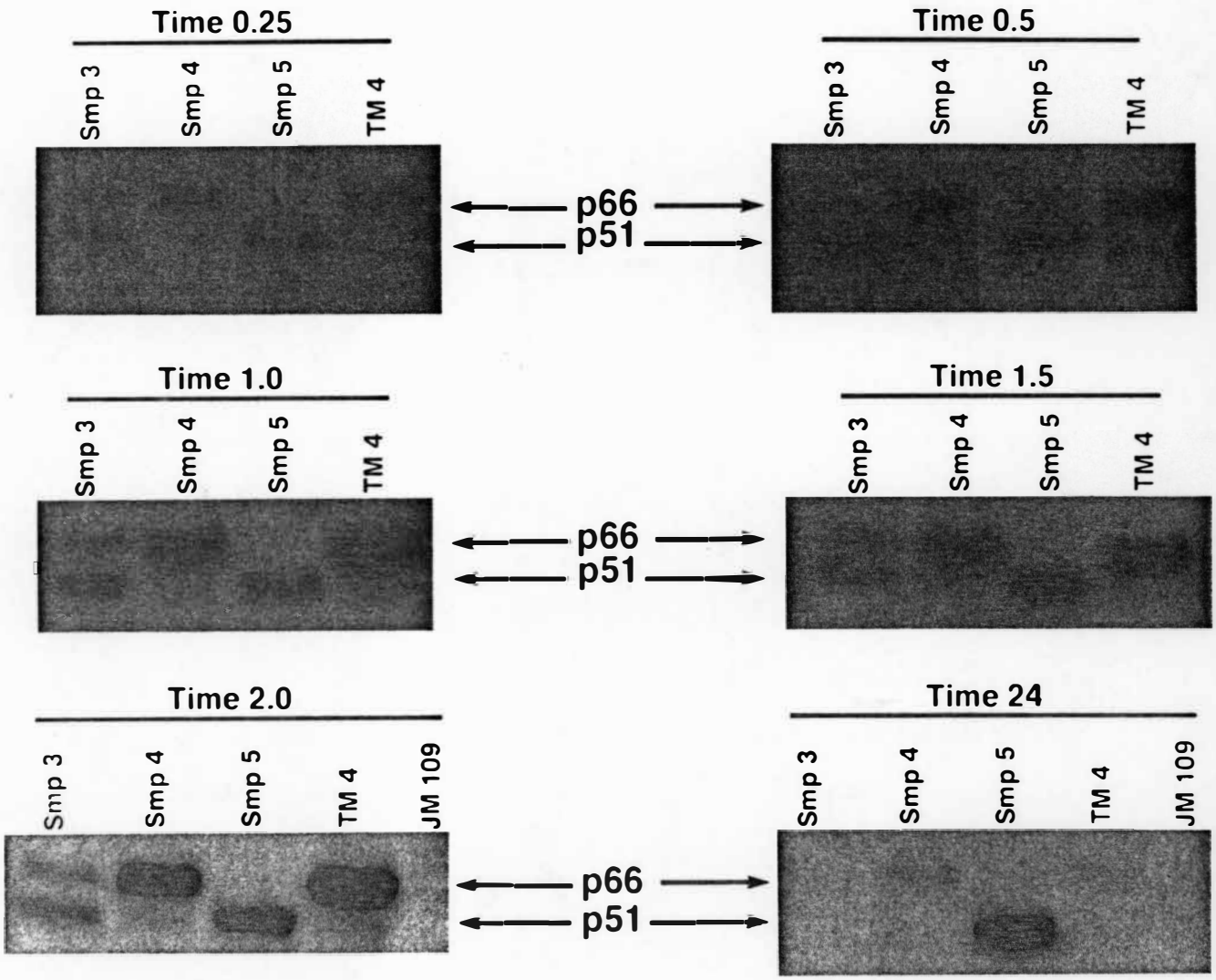

Figure 4. Autoradiograph from western blot on samples harvested 0.25, 0.5, 1.0, 1.5, 2.0, and 24 hours post-induction. Both forms of RT (p66 and p51 KDa proteins) were found as shown for the mutants Smp 3, 4, and 5 and native RT (TM4). These proteins were not found in the JM109 (host cells without plasmid) samples evaluated at the 2.0 and 24 hour time points.

Discussion

Both mutants Smp3 and Smp4 produced proteins which acted like the native form (TM4) enzymatically and electrophoretically (Table 2 and Figure 4), indicating that the amino acid change was well tolerated. However, mutant Smp3 produced the p51 form at an earlier time point after induction than did TM4, suggesting that the processing event was accelerated. Although direct evidence is lacking, the change from a negatively charged amino acid (glutamic acid) to an uncharged amino acid (glutamine) may have created a conformational change in the 66 KDa protein making it more readily accessible to proteolytic or autocatalytic cleavage events. Therefore, although the amino acid changes made in the mutants were well tolerated, the slight differences in results observed, compared to the results of native RT, suggests that the amino acid glutamine is critical to the processing site.

Comparison of the polypeptides seen on the autoradiographs to RT enzymatic activity indicate that the p66/p51 heterodimer form is the most active. This is based on the fact that RT enzymatic activity is higher for mutant Smp3 than either mutant Smp4 or TM4 at time point 0.25 hour after induction (Table 2). The western blot procedure indicated the presence of both p66 and p51 for Smp3 while only the p66 protein was visible for Smp4 and TM4 at this same time point. However, between time points 0.5 and 1 hour the presence of a p51 polypeptide as well as the p66 polypeptide was seen in all three of these preparations which correlates to the comparable enzymatic activity seen at these same times.

The p66 form of RT also appears to be enzymatically active (although less than the p66/p51 form) since substantial RT activity was measured at all time points when the p66 polypeptide alone was found using the western blot (Smp4 and TM4). This conclusion agrees with Starnes et al. (1988) who reports finding the p66 polypeptide of RT to be

sufficient for both DNA- and RNA-directed DNA synthesis in an assay similar to the one used in the project.

Lastly, Smp 5 produced only the p51 form of RT which showed low levels of RT enzymatic activity even when present in sufficient quantities as seen on the western blots (Figures 3 and 4). Hizi and coworkers (1988) used deletion mutagenesis to produce a RT (51 KDa) protein. This protein lacked the carboxyl terminus (133 amino acids) of the p66 form and showed little enzymatic activity. Perhaps the Smp5 mutant with the change in two amino acids at the proposed processing site increased p66 processing to the point where only the p51 form is found at all time points evaluated.

The RT enzymatic properties found for the mutants and native RT as described above suggest and are in agreement with others (Lowe et al., 1988; Deibel, et al., 1989) that the p66/p51 heterodimer is the most active, the p66 homodimer the second most active, and the p51 homodimer the least active form of HIV-RT.

In conclusion, site-directed mutagenesis and evaluation of the mutants resulting products as done in this research project has proven to be a viable strategy for the study of HIV-RT p66/p51 processing. The differences found between the mutants and native RT both enzymatically and electrophoretically provide evidence in support of the idea that they were made at or near the proposed processing site.

Appendix A

List of Reagents and Media

LIST OF REAGENTS AND MEDIA

REAGENTS

Kinase buffer A: 0.1M Tris-HCl, pH 8.0, 10mM $MgCl_2$, 7mM dithiothreitol, & 1mM ATP.

Kinase buffer B: 1M Tris-HCl, pH 8.0, 100mM $MgCl_2$, & 70mM dithiothreitol.

Ligase buffer: 50mM Tris-HCl, pH 7.8, 10mM $MgCl_2$, 20mM dithiothreitol, & 50 micrograms/ml bovine serum albumin.

NETG: 150mM NaCl, 5mM EDTA, 50mM Tris-HCl, pH 7.4, & 0.25% gelatin, 60 bloom.

RT buffer: 20mM dithiothreitol, 60mM NaCl, 0.05% NP-40, 10mM $MgCl_2$, & 50 mM Tris-HCl, pH 8.3.

2X sample prep buffer: 20% w/v glycerol, 10% v/v 2-mercaptoethanol, 4.6% w/v SDS, & 0.125M Tris-HCl, pH 6.8.

30X SSC: 4.5M NaCl & 0.45M trisodium citrate.

STET buffer: 8% sucrose, 5% triton X-100, 50mM EDTA, & 50mM Tris-HCl, pH 8.0.

TBE buffer: 89mM Tris-borate, 89mM boric acid, & 2mM EDTA.

TE buffer: 10mM Tris-HCl & 1mM EDTA, pH 8.0.

TEN_{100}: 50mM Tris-HCl, pH 8.0, 10mM EDTA, & 200mM NaCl.

MEDIA

Glucose/minimal medium plates: M9 salts, 1M $MgSO_4$, 1M thiamine-HCl, 0.1M $CaCl_2$, 20% glucose, & 1.5% agar.

H top agar: 1.0% Bacto tryptone, 0.8% NaCl, & 0.8% agar.

H plate: 1.0% Bacto tryptone, 0.8% NaCl, & 1.5% agar.

Luria-Bertani medium: 1% Bacto tryptone, 0.5% Bacto yeast extract, & 1.0% NaCl.

2X TY medium: 1.6% Bacto tryptone, 1% Bacto yeast extract, & 0.5% NaCl.

2X TY agar: 2X TY medium with 1.5% agar.

Appendix B

List of Suppliers

LIST OF SUPPLIERS

Amersham, Arlington Heights, IL

Bacto, Detroit, MI

Bethesda Research Laboratories, Gaithersburg, MD

Bio-Rad, Richmond, CA

Carnation, Los Angeles, CA

Eastman Kodak Co., Rochester, NY

Enprotech, Hyde Park, MA

Fluka Chemie, Switzerland

FMC BioProducts, Rockland, ME

Hoefer Scientific, San Francisco, CA

Irvine Scientific, Santa Ana, CA

New England Biolabs, Beverly, MA

New England Nuclear, Wilmington, DE

Promega, Madison, WI

QIAGEN, Studio City, CA

Savant, Farmingdale, NY

Schleicher & Schuell, Keene, NH

Sigma, St. Louis, MO

Waters Associates, Milford, MA

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