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THE EFFECT OF NUCLEOSIDE ANALOGUES ON HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 INFECTION

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Dr Eric Arts is responsible for the design and implimentation of the experiments that resulted in Figures 9 and 10 of this thesis. Dr. Z Gu is responsible for the creation of all recombinant clones that were used in the experiments in this thesis.

I like to dedicate this thesis and pledge my continued commitment to working towards bringing an end to the AIDS pandemic to the memory of Rodney Garbato, dear departed friend, who died of complications associated with AIDS in 1989.

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Abstract

Human immunodeficiency virus type-1 (HIV-1) reverse transcriptase (RT) is essential for the formation of the viral double-stranded DNA genome from its RNA template. Blockage of this catalytic function by reverse transcriptase inhibitors leads to inhibition of viral replication. Although 3'-azido-3'-deoxythymidine (AZT) is the most successful anti-viral drug to date, its prolonged use results in the emergence of AZT-resistant viral isolates. Several mutations are consistently found within the reverse transcriptase gene of AZT-resistant viral isolates. Although it is generally accepted that these mutations are responsible for the drug-resistance phenotype, the exact mechanism by which this occurs is poorly understood. For example, cloned viruses harboring these mutations show resistance to AZT in tissue culture, yet recombinant HIV RTs that contain these resistance-conferring "mutations" do not show increased inhibition constant (K_i) values for AZT as compared to wild type RTs.

By *in vitro* infection, polymerase chain reaction , and reconstituted *in vitro* reverse transcription assays, we have confirmed previous observations (Arts 1994c) that AZT preferentially terminates HIV-1 nucleic acid elongation after the first template switch. We have also shown that nucleoside analogues are more effective chain terminators in situations in which deoxynucleotide triphosphate pools are limiting. Pre-treatment of cells with AZT prior to infection had a stimulatory effect on

generation of viral DNA, but only in the case of AZT-resistant variants and not wild-type strains of HIV-1.

Résumé

La transcriptase inverse (TI) du virus de l'immunodéficience humaine de type 1 (VIH-1) est essentielle à la formation du génome d'ADN viral double brin à partir de sa matrice d'ARN. Le blocage de cette fonction, par des inhibiteurs de la TI, mène à l'arrêt de la réplication virale. Même si le 3'-azido-3'-deoxythymidine (AZT) est la drogue anti-virale la plus efficace jusqu'à maintenant, son usage prolongé entraîne l'apparition de virus résistant à l'AZT. Plusieurs mutations sont continuellement retrouvées dans le gène de la TI des différents virus résistants à l'AZT. Même s'il est généralement accepté que ces mutations sont responsables du phénotype de résistance, le mécanisme précis par lequel cela se produit est peu compris. Par exemple, des clones de virus ayant ces mutations sont résistants à l'AZT en culture de tissus. Cependant, les TI recombinées du VIH-1 contenant ces mutations conférant la résistance ne présentent pas d'augmentation de la constante d'inhibition (K_i) de l'AZT comparées aux TI de type sauvage.

À l'aide d'infection *in vitro*, de réaction de polymérisation en chaîne et de réactions reconstituées de la TI *in vitro*, nous avons corroboré les observations antérieures affirmant que l'AZT arrête préférentiellement l'élongation de l'acide nucléique du VIH-1 après le premier changement de matrice (template switch). Nous avons aussi démontré que les analogues nucléosidiques sont meilleurs pour arrêter l'élongation lorsque la concentration de déoxynucléotide triphosphate est limitée. Le

pré-traitement des cellules avec l'AZT, avant l'infection, avait un effet stimulant sur la production d'ADN viral, mais seulement pour les virus résistants à l'AZT et non pour les souches sauvages du VIH-1.

Introduction

A. HIV-1 Life Cycle

The human immunodeficiency virus type-1 (HIV-1) infects and destroys CD4+ cells of the immune system (Maddon 1986, McDougal 1986, Hwang 1991) inducing a slow degenerative disease known as acquired immunodeficiency syndrome (AIDS). The virus has two functionally active single stranded (ss) (+) RNA genomes that code for 9 viral genes (*gag, pol, env, vpr, vpu, vif, nef, rev,* and *tat*). Upon binding to the surface of an infected cell, the viral core is endocytosed into the cytoplasm following a direct pH-independent fusion with the plasma membrane that involves interaction between the cell surface CD4 and viral gp120/41 envelope glycoproteins (Stein 1987). Cellular deoxynucleotide-5'-triphosphates (dNTPs) enter the viral core upon its partial dissolution and the ss (+) RNA genome is converted into double stranded (ds) proviral DNA by the HIV-1 protein reverse transcriptase (RT) (Gilboa 1979, Weiss 1985). This ds proviral DNA is actively transported to the nucleus within an integration complex that consists of the HIV-1 proteins integrase (IN), nucleocapsid (NC), and matrix (MA) (Bukrinsky 1993 and 1992). HIV-1 IN then non-specifically orchestrates integration of the proviral DNA into chromosomal DNA. Once integrated, the viral long terminal repeat (LTR) binds host cellular transcription factors and regulates the transcription of HIV-1 genes (Cobrinik 1988, Engelmann 1991, Murphy 1989). The first genes expressed are the

doubly spliced regulatory genes that encode the tat, nef, and rev proteins.

HIV-1 tat is a transcriptional transactivator that increases expression of HIV-1 genes (Arya 1985, Lespia 1989, Shapr 1989, Sodroski 1985). Nef down-regulates the cell surface expression of CD4 and plays a possible role in the pathogenesis of HIV-1 infection (Aiken 1995 and 1994, Kestler 1991). Rev regulates expression of viral proteins by allowing singly spliced (env, vif, vpr, vpu) and unspliced (gag, gag/pol) proteins to be expressed in the cytoplasm (Hadzopolou-Cladares 1989, Malim 1989, Sodroski 1985).

The envelope protein is expressed as a gp160 glycoprotein precursor that is cleaved by the cellular protease, furin, into gp120 and gp41 which associate non-covalently at the cell surface and await the rest of the viral particle prior to virus budding and maturation (Earl 1991, Hallenberger 1992). Vif is a non-structural cytoplasmic protein that is thought to be an infectivity factor and has been shown to be absolutely required for infection of peripheral blood mononuclear cells (PBMC) (Gabudza 1992, Sakai 1993, VonSchwelder 1993). Vpr is a nuclear protein that is incorporated into the budding virus and is thought to play a role in viral production. It may also be involved in the active transport of the preintegration complex to the nucleus of an infected cell (Cohen 1990, Heinzinger 1994, Levy 1993, Paxton 1993). Vpu is thought to play a role in CD4 degradation, virus particle release, and in the regulation of virally induced cytopathic effect (CPE) (Gottlinger 1993).

Gag encodes for the p55gag polyprotein precursor that is cleaved upon viral budding by the viral protease into p15 (NC), p17 (MA), and p24 (CA) structural proteins. NC (p15) is a basic polyprotein that non-specifically binds nucleic acid and is associated with genomic RNA in the virion. It is essential for production of infectious virus and is further cleaved by the viral protease into NCp7, NCp1, NCp2, and NCp6 proteins. NCp7 coats the viral RNA, protecst it from nucleases, and promotes reverse transcription (DeRocquigny 1992, Henderson 1992). MA (p17) is a myristylated protein that exists in close association with the viral membrane or envelope. It has a nuclear localization signal (NLS) and plays a role (along with vpr) in the active transport of the HIV-1 preintegration complex into the nucleus (Bukrinsky 1993, Bukrinsky 1992, VonSchwedler 1994). CA (p24) is a hydrophobic structural protein that constitutes the major internal structural feature of the virion core/shell. It forms a bullet-shaped core that surrounds the mature virion (Haseltine 1991).

p160gag/pol fusion protein results from an infrequent (-)1 ribosomal frameshift that occurs about 10% of the time during translation of the p55gag polyprotein and results in a readthrough of a stop codon in the p55gag protein (Jacks 1988, Parkin 1992). The p160gag/pol polyprotein precursor encodes NC, MA, and CA proteins as well as the viral genes protease (PR or p11) , integrase (IN or p32), and reverse transcriptase (RT or p66/51). PR (p11) is a homodimeric protein that is responsible for the cleavage and subsequent maturation of viral proteins. IN (p32) is responsible for integrating the ds proviral DNA non-specifically into the host chromosome. RT

(p66/p51) is a heterodimeric protein that is responsible for the conversion of viral ss (+) genomic RNA into ds proviral DNA. Once all of these viral proteins are expressed (RT, PR, IN, NCp15, vpr, ds (+) RNA), they, along with certain other cellular components such as $tRNA^{Lys,3}$ and cellular cyclophilins, assemble beneath the plasma membrane and immature viral particles bud from the cell surface (Barat 1993, Berkowitz 1993, Gottlinger 1989, Lavallee 1994, Mak 1994, Murphy 1989, Sakaguchi 1993). HIV-1 (a type-D retrovirus) buds from the infected cell membrane non-cytopathically as an immature form. At some uncertain point during this assembly and budding process, the viral protease cleaves itself) and then goes on to cleave all of the other viral proteins generating a mature viral particle that can infect new cells. Dimeric RNA undergoes maturation after the virus has been released from the cell and then condenses to a more compact conformation (DiMarzo-Veronese 1986, Fitzgerald 1992, Henderson 1992, Kohl 1988, LeGrice 1988, Zybarth 1994).

B. HIV-1 Pathogenesis

The first cases of AIDS were reported in 1981 (Gottlieb 1981). HIV-1 was first isolated and characterized in 1983 (Barre-Sinoussi 1983, Popovic 1984). HIV-1 has since been shown to be the cause of AIDS.

HIV-1 uses its gp120 surface glycoprotein to infect cells that express the cell surface marker CD4. Although the CD4+ T-helper cell is probably the prime target of HIV-1 infection, HIV-1 tropism is not as restricted as was once thought to be the case. HIV-1 is also

capable of infecting B cells, thymocytes, neurons, colorectal cells, monocyte/macrophages, brain monocyte/macrophages, dendritic cells, eosinophils, hepatocytes, capillary endothelial cells, glial cells, and CD34+ bone marrow derived precursor cells (Gelezuinas 1993, Mercure 1994).

HIV-1 infected cells display functional defects. Progression of HIV-1 disease is marked by a gradual and significant decrease in numbers of CD4+ T helper lymphocytes. Initially, it was thought that only very low numbers (0,1 to 1%) of peripheral blood CD4+ T lymphocytes were productively infected during early and intermediate stages of HIV-1 infection, (Harper 1986, Schnittman 1989). However, discrepancy existed between the proposed low numbers of infected CD4+ T cells and the much larger numbers of CD4+ T cells that were being destroyed (Brinchmann 1991). It was thought that decreases in CD4+ numbers might result from indirect mechanisms of depletion such as apoptosis, antibody-dependent cellular cytotoxicity (ADCC), syncytium induction (SI), or cell mediated immunity (CMI) (Ameisen 1992, Choi 1991, Clerici 1993, Smith 1993a). Recent evidence, however, suggests that the infection level of CD4+ T cells is much greater than previously believed (Ho 1995, Wei 1995). The percentage of infected CD4+ lymphocytes found in lymphoid tissues, e.g. lymph nodes, is far higher then the 0,1-1% levels reported for PBMCs (Embretson 1993, Pantaleo 1993). Furthermore, a quantitative-competitive PCR technique has now shown that high levels of HIV RNA are present in peripheral blood of infected individuals (Piatak 1993).

HIV-1 infection provokes an immune response that is thought to be generated in lymph nodes in which HIV-1 is initially contained by follicular dendritic cells, which themselves, may not be susceptible to HIV-1 infection (Fox 1991, Pantaleo 1993,). Initially, an effective immune response is generated by the host in response to infection by HIV-1. This response includes both a neutralizing antibody and specific cytotoxic T cell response (Clerici 1993, Wainberg 1993). The virus, however, continues to replicate. It is thought that for a significant portion of the period of HIV-1 infection, the number of virus particles destroyed by the immune system each day may be equivalent to the number of new virions produced (Ho 1995, Wei 1995).

The immune system is incapable of clearing HIV-1 because of the rapid rate of viral replication and the high mutation rate of HIV-1 (Hahn 1986, Preston 1988, Roberts 1988). Infected persons harbour a wide array of quasi-species of HIV-1 simultaneously. This concept is central to HIV-1 pathogenesis and the mechanisms whereby HIV-1 evades immune attack. As HIV-1 infection progresses, CD4 counts diminish, lymph nodes degenerate (follicular dendritic cells are no longer able to contain the virus), and the body becomes less able to mount effective immune responsiveness leading to opportunistic infection (OI). The latter include neurological disorders (myelopathy, peripheral nerve disease, meningitis, encephalopathy, AIDS dementia), wasting, and infections due to bacterial, fungal, parasitic and other viral (EBV, CMV, HSV) causes.

C. HIV-1 Reverse Transcriptase

Reverse transcriptase (RT) was discovered independently by Howard Temin (RSV RT) and David Baltimore in 1970 (MuMLV RT). To date, reverse transcriptase activity has been documented in telomeric DNA of nearly all eucaryotic cells, in retrotransposons, in bacteria such as *Myxococcus xanthus* and *Eschericia coli*, in hepadnaviruses, in cauliflower mosaic virus, and, in all retroviruses, including HIV-1 (Dhundale 1987, Guilley 1983, Inouye 1989, Pfeiffer 1983).

HIV-1 RT is the viral enzyme (polymerase) responsible for the conversion of ss (+) RNA viral genome into ds proviral DNA. The enzyme can exist in many different forms; a p160gag-pol precursor, a p66 RT monomer or homodimer (association constant (K_a) of 2,3 x 10^5 $M^{-)}$, a p51 RT monomer or homodimer (K_a of 1,3 x 10^3 $M^{-)}$, and a p66/p51 RT heterodimer (K_a of 10^9 $M^{-)}$ (Restle 1990). All of these forms are capable of polymerase activity (Hansen 1988, Starnes 1989), but only the heterodimer (and to lesser extent, the p66 homodimer) are highly efficient (Davies 1991, Hostomosky 1991).

The heterodimer has been crystallized. It is the only form that is actually found in virions. It is very stable (half-life ($t_{1/2}$) at 0'C of greater that 1000 hours), and suffices for complete proviral DNA synthesis (DiMarzo-Veronese 1986, Jacob-Molina 1991, Larder 1987b, Lowe 1988, Restle 1990).

HIV-1 RT is synthesized as a p66 homodimer that undergoes cleavage to become a p51/p66 heterodimer. The p66 subunit is roughly 560 amino acids long. It is well conserved in evolution closely resembling murine Moloney leukemia virus (MuMLV) RT,

avian leukemia virus (ALV) RT, and equine infectious anemia virus (EIAV) RT (Borroto-Esoda 1991, LeGrice 1991, Ruprecht 1986). It is folded in a precise 2' and 3' structure that is required for proper enzymatic function. The p51 subunit is the viral protease cleavage product of the p66 subunit. Because essential catalytic residues are buried within the p51 subunit, it has only little catalytic activity (DiMarzo-Veronese 1986, LeGrice 1989, Mizrahi 1989). It is thought that the p51 subunit probably contributes to the process of polymerization by maintaining or establishing the active conformation of the p66 subunit in the heterodimer. The portion of HIV-1 RT that is cleaved from the p66 subunit to form p51 subunit is a p15 subunit, that contains the enzyme's RNaseH domain. However, p15 is incapable of RNaseH activity and its function in isolated form is unknown (Beccera 1990, Schatz 1989, Tisdale 1988).

The p66 subunit has been likened structurally to a human right hand with five subdomains (palm, thumb, fingers, connection, and RNaseH) that are involved in grasping a primer and a template (P/T) complex (see Figure 1). The palm region (amino acids 85-119, and 151-244) contains the catalytic site for polymerization. The fingers region (amino acids 1-62, 63-84, 120-150) is attached to the palm and faces the primer template binding pocket and is thought to contain the dNTP binding site. The thumb region (amino acids 245-322) is thought to recognize and bind the primer. It has been shown to be necessary for primer binding and is situated perpendicular to the connection subdomain. The connection region (amino acids 323-437) links the polymerase and RNaseH domains. The RNaseH region (amino acids 438-560) trails the polymerase region by 18-22

nucleotides along the nascent nucleic acid and is responsible for digestion of the genomic RNA template as proviral DNA is synthesized (Davies 1991).

The fingers, palm, and thumb subdomains together constitute the polymerase region. Amino acids 183-186 (YMDD), found within the palm subdomain, constitute the actual proposed *pol* active site of HIV-1 RT (Larder 1987a). This motif is semi-conserved in nearly all cellular and viral RNA-dependent RNA polymerization (RDDP) and DNA-dependent polymerization (DDDP) (Argos 1988, Donahue 1988, Inouye 1989). The YMDD in HIV-1 is absolutely essential for RT function and cannot, with one important exception, be mutated (Boyer 1992a, Larder 1989a and 1987a, Prasad 1989). Mutations of M184 to either V or A, however, generate a virus that has wild type reverse transcriptase activity, is completely replication competent and is resistant to the nucleoside analogues ddI, ddC, and 3TC (Gao,Q. 1993a and 1993b, Gu 1992, Schinazi 1993).

The HIV-1 RT polymerase active site forms a binding pocket for divalent cations that is analogous to the pol I active site of *E. coli* (Polesky 1992 and 1990). The YMDD residues D185 and D186 are found in a short loop in close proximity to the D110. Together, these D residues interact with Mg^{2+} or Mn^{2+} and are essential for nucleophilic attack by the primer's free 3'-OH on the α–phosphate of the incoming deoxynucleotide triphosphate (dNTP) (Kohlstaedt 1992). Other residues in the HIV-1 RT that are important for the pol active site include D113, A114, Y115, Q155, K154, Y181, Y183, and M184. These residues are not directly involved in Mg^{2+} binding, but may be

important for the structural integrity of the active site (Boyer 1992a and 1992b, Kohlstaedt 1992, Larder 1987a and 1989a).

The HIV-1 RT dNTP binding site has been roughly mapped to amino acids 65 to 73 (Boyer 1992b, Cheng 1993, Wu 1993). These amino acids are found either flanking or actually in the fingers subdomain of RT opposite the primer/template binding site on the thumb subdomain and in a position to drop a bound dNTP into the polymerization active site in the palm (Jacob-Molina 1993, Kohlstaedt 1992). Amino acid 73 is thought to be the binding site for dTTP (Cheng 1993). Only the p66 subunit in the HIV-1 p66/p51 heterodimer binds dNTPs (Cheng 1993).

D. HIV-1 Reverse Transcription

Retroviral replication involves the conversion of ss (+) RNA viral genome to a ds proviral DNA copy (see Figure 2). This process involves: two priming events involving a host tRNA species and an RNaseH-resistant region of viral genomic RNA known as the poly purine tract (PPT); two template switches which involve the transposition or jumping of DNA from one template to another; RNA-dependent RNA polymerization (RDDP); DNA-dependent DNA polymerization (DDDP); and RNaseH and RNaseD removal of viral RNA and tRNA primers. *In vivo*, HIV-1 reverse transcription occurs within a transcription complex that consists of a host $tRNA^{Lys,3}$ primer, HIV-1 RT, HIV-1 NC protein, genomic RNA, and possibly other viral and cellular factors (Barat 1989, Leis 1993).

Within the transcription complex, host $tRNA^{Lys,3}$ binds to the primer binding site, i.e. an 18 nucleotide region of the HIV-1 DNA that is situated between the 5' LTR and the gag gene and is perfectly complementary to the 3' end of host $tRNA^{Lys,3}$. This event primes RDDP which is the first step of the reverse transcription process. It is unclear whether this event occurs in the immature virus during assembly, in the virion after budding and/or in the host cell upon core entry, since short, early DNA products of reverse transcription have been documented within virions (Arts 1994a, Biswal 1971, Levinson 1970, Lori 1992, Zhang 1993).

The first species of HIV-1 DNA that is reverse transcribed is called (-) strong-stop DNA. It is covalently linked to $tRNA^{Lys,3}$ and represents the complement of U5 and R. It is released as a single strand of DNA as the RNA template is exo- and endo-nucleolytically digested by the RNaseH activity of the HIV-1 RT (DeStephano 1991a and 1991b, Schatz 1990).

Once the R region of (-) strong-stop DNA has been freed of its RNA template by RNaseH, a process known as the first template switch ensues, whereby (-) strong-stop DNA is translocated from the 5' end of (+) viral RNA to the 3' end of the same or a second viral RNA molecule because of complementarity between the 5' and the 3' R regions (Luo 1990, Oyama 1989, Panganiban 1988, Peliska 1992). The first template switch may occur either intra- or inter-molecularly (i.e., between the same or different RNA molecules, (Panganiban 1988). This (-) strong stop DNA now annealed to the 3' R then serves as a primer to continue (-) strand DNA synthesis and RNaseH digestion of the template (Hu 1990). RNaseH digests the

entire RNA template except for a region known as the polypurine tract (PPT) which consists of at least 9 purine (Adenine and Guanine) residues located immediately preceding the U3. Its resistance to RNase digestion is thought to be due either to its secondary structure or its specific sequence (Champoux 1984, Omer 1984, Resnick 1984, Smith 1984a and 1984b). This PPT serves as the primer for synthesis of (+) strand viral DNA (Omer 1984). (-) HIV-1 DNA serves as the template in the DDDP synthesis of (+) strand HIV-1 DNA. The $tRNA^{Lys,3}$ primer remains annealed to the PBS and requires RNaseD cleavage to get rid of the HIV genomic RNA (BenArtzi 1992, Roth 1989). Liberated $tRNA^{Lys,3}$ then serves as a template for the synthesis of new (+) strand DNA.

(+) strand DNA synthesis continues from the PPT primer and appears to terminate after copying a region of the $tRNA^{Lys,3}$. For $tRNA^{Lys,3}$ to act as a template in (+) strand DNA synthesis, RT must first switch from RNaseH to RNaseD activity to digest the PBS RNA in the PBS/tRNALys,3 RNA:RNA duplex , then switch from DDDP to RDDP (Roth 1989, BenArtzi 1992, Omer 1984). The first modified base on the $tRNA^{Lys,3}$ is thought to serve as a precise stop position for HIV-1 RT DDDP (Roth 1989). The $tRNA^{Lys,3}$ primer template is then removed by RNaseH digestion which is initiated between terminal adenosine and cytidine bases of $tRNA^{Lys,3}$ (Smith 1992, Whitcomb 1990). This is essential for further extension of (+) DNA after the second template switch.

The latter results in the transfer of a short (+) DNA from the 5' end of the (-) DNA to the 3' end of the same or another (-) strand (Panganiban 1988). There is controversy surrounding this but it has

been postulated that only 1 (-) DNA is used as a template for the generation of (+) DNA, i.e., the second template switch is an intrastrand switch (Panganiban 1988). Regardless whether the second template switch is inter- or intra-strand, the binding of (+) strong stop DNA to the PBS of the (-) DNA can prime DDDP for the completion of (+) DNA synthesis and the generation of double stranded proviral DNA.

Concomitant with this process of nucleic acid anabolism, HIV-1 RT carries out both endonucleolytic and 3' -> 5' exonucleolytic RNase H activity (Tan 1991) which removes genomic RNA after it has served as a template for the synthesis of proviral DNA (Leis 1973). HIV-1 RT RNaseH and polymerase activities are tightly coupled processes spatially but are functionally independent. The pol active site is separated from the RNaseH domain by at least 50 Angstrom, which corresponds to about 15-16 bases of dsDNA (Arnold 1992, Jacob-Molina 1993, Lederer 1992). Different RTs (avian, murine) have different spatial separations between their polymerase and RNaseH domains (Oyama 1989). The model proposed is that a relatively small amount of DNA is synthesized followed by cleavage of the RNA template by RNaseH. Cycles of synthesis and cleavage ensue until completion of the DNA strand (Oyama 1989).

It is not entirely certain when reverse transcription is actually initiated since it has been documented that incomplete viral DNA is carried into a newly infected cell by an invading virion. The existence of this virion-associated DNA species is not required for infection (Arts 1994a), but reverse transcription is initiated in virions prior to host cell entry (Gao,W-Y 1993a, Lori 1992, Zhang 1993).

The process of reverse transcription can be reconstituted *in vitro* with primer and template (P/T), dNTPs, recombinant or purified RT in either the presence or absence of NC protein. Recombinant HIV-1 RT can be expressed using several different systems: bacterial, insect, and plasmid expression vectors (Farmerie 1987, Larder 1987b, Hansen 1988, LeGrice 1988). The mode of purification of HIV-1 RT can be carried out by several means: HPLC size exclusion chromatography; immunoaffinity chromatography; and by metal chelate affinity chromatography, known as the histidine tag method (LeGrice 1990, Restle 1990). It must be noted that different methods of HIV-1 RT expression and purification result in significant differences in enzymatic activity (Martin and LeGrice personal communications). Purified and recombinant RT can be used in hetero- or homo-polymeric, natural or synthetic, DNA or RNA P/T combinations (poly(rA):oligo$(dT)_{12-18}$, activated calf thymus DNA, poly(rI):oligo $(dC)_{12-18}$, poly(rC):oligo$(dG)_{12-18}$, poly(rCm):oligo$(dG)_{12-18}$.) The template that works best is poly(rA): oligo$(dT)_{12-18}$ (Hoffman 1985, Rey 1987, St.Clair 1987).

E. Nucleoside Analogues as Treatment for HIV-1 Infection

Reverse transcriptase inhibitors known as 2',3'-dideoxynucleoside analogues (ddNTPs) are the most utilized and researched of all anti-HIV-1 therapeutic agents. Zidovudine, or AZT, is the prototype of these RT inhibitors. 3TC, ddI, and ddC are three other ddNTPs currently used to treat HIV-1 infection.

The use of AZT has been shown to increase survival in patients with advanced HIV disease and to delay progression in patients with mild/symptomatic disease. However, AZT therapy has not been demonstrated to have beneficial effects on disease progression or survival in patients with asymptomatic infection (Basham 1990, Hirsch 1990, Wilde 1993). Use of AZT has been shown to improve neurological and immunological function, to increase the number of circulating CD4+ cells; to decrease viral load, and to lead to weight gain, less fatigue, and a general sense of wellness (Richmann 1987a, VanLeeuwen 1992, Wilde 1993, Yarchoan 1989 and 1986).

The use of nucleoside analogues, however, is sometimes associated with severe toxicity, and beneficial effects may be short-lived, probably due to resistance that develops to these compounds. AZT has been shown to cause bone marrow suppression, nausea, myalgia, insomnia, headaches, transient agitation, vomiting, macrocytosis, anemia, elevated platelet numbers, leukopenia and neutropenia. ddC and ddI are both associated with peripheral neuropathy and ddI is further associated with pancreatitis. Patients who are in more advanced stages of disease are more likely to suffer haematologic side effects (Richmann 1987a, Yarchoan 1989). These side effects are usually dose-dependent and disappear or resolve spontaneously when drug doses are decreased or drug is discontinued (Wilde 1993). 3TC is the least toxic of the nucleoside analogues studied, followed by ddI, AZT, and ddC (Coates 1992, Hart 1992, Pauwels 1992).

Nucleoside analogue toxicity may arise due to effects on host cellular DNA polymerases (DNA polymerases α, β, γ, and δ) (Furman

1986, Parker 1991). HIV-1 RT affinity for nucleoside analogues is 100 - 1000 times greater than that of cellular polymerases (Balzarini 1989, Parker 1991, St.Clair 1987, Yarchoan 1989). Nevertheless, some cellular DNA polymerases are more susceptible to the effects of nucleoside analogues then others. For example, mitochondrial DNA polymerase γ is the most susceptible cellular polymerase to physiologically relevant concentrations of AZT. Nucleoside analogue toxicity may also arise from cell specific alterations induced in cellular dNTP pools, which may effect the cell's biochemistry (Cox 1992a). A further complication associated with the use of nucleoside analogues in the treatment of AIDS is that in patients, drug pressure can select for resistant variants, generated, in part, because of high viral mutation rate (Wainberg 1993, Wood 1987).

Nucleoside analogues must be triphosphorylated to have activity. They first permeate membranes by either facilitated (ddI, ddC) or non-facilitated (AZT) diffusion (Kong 1992). Once inside the cell they are phosphorylated by cellular kinases, nucleotidases and other activating enzymes to their active triphosphates. These enzymes are present naturally within cells and are differentially expressed dependent on host species, cell type, and stage in cell cycle (Cox 1992a, Somadossi 1993, Yarchoan 1989). Expression of these enzymes is generally not altered by HIV-1 infection (Cox 1992a, Szebeni 1991). Each nucleoside analogue requires a different phosphorylation pathway. Figure 3 depicts the phosphorylation pathways for two different nucleoside analogues, AZT and ddI.

Phosphorylation of AZT occurs via the thymidine salvage pathway (Cox 1992a, Furman 1986). AZT is phosphorylated to its

monophosphate derivative (AZT-MP) by the cellular enzyme thymidine kinase (TK) (Furman 1986, Mitsuya 1987, Yarchoan 1989). AZT-MP accumulates intracellularly to high levels and has been shown to be the rate limiting step in AZT triphosphorylation (Balzarini 1989). AZT-MP is phosphorylated to a diphosphate (AZT-DP) by the cellular enzyme thymidylate kinase (Mitsuya 1987, Yarchoan 1989). AZT-DP is then phosphorylated to the triphosphate (AZT-TP), again by thymidylate kinase (Mitsuya 1987, Yarchoan 1989). The phosphorylation of AZT-MP to AZT-DP and AZT-TP occurs at a 200-600 fold lower level than that of AZT to AZT-MP. Hence, AZT-DP and AZT-TP are present intracellularly at lower levels than AZT-MP (Balzarini 1989). Intracellular concentrations of AZT-TP are commonly between 1 and 7 μM, i.e., between 25 and 160 times higher than the inhibition constant (K_i) for RT, i.e., 0,04 μM (Furman 1986).

The main mechanism whereby AZT and other nucleosides inhibit HIV-1 replication is by causing chain termination. These compounds lack a 3'-hydroxyl group, necessary for 5' to 3' DNA chain elongation. Hence, these ddNTPs cause chain termination when they are incorporated in place of the native substrate (Yarchoan 1989).

AZT and other nucleoside analogues also inhibit HIV-1 replication by competitive inhibition with native nucleoside triphosphates. For example, AZT-TP competes well with dTTP for binding to the active site of HIV-1 RT (Cheng 1987, Furman 1986, St.Clair 1987).

Nucleoside analogues can perturb dNTP pools, leading to cellular toxicity (see above). This toxicity may sometimes lead to

indirect inhibition of HIV-1 replication. Intermediate metabolites of AZT may also exert anti-viral activity. AZT-MP can inhibit both RDDP and RNaseH, and can also block HIV-1 DNA integration, albeit at very high concentrations (Tan 1991). AZT or its derivatives may also act post-integrationally by interfering with assembly and release of progeny virus (Rooke 1990). The use of AZT in tissue culture has also been shown to decrease the ability of uninfected cells to participate in HIV-induced syncytium formation (Buckheit 1992).

F. HIV-1 Drug Resistance

Drug-resistant strains of HIV-1 can be isolated from patients who have been treated with AZT for 36 or more weeks (Basham 1990, Land 1992, Larder 1989c, Wainberg 1993, 1992, and 1990). AZT-resistant HIV-1 has also been isolated from patients never treated with AZT, suggesting that drug-resistant forms may be sexually transmitted (Mohri 1993, Wainberg 1990, Wilde 1993). Drug resistant viruses can be selected *in vitro* through gradual increases in drug concentration (Larder 1991, Gao, Q. 1992 and 1993b). In some cases, drug-resistant viruses were identified on the basis of tissue culture selection, before equivalent viruses were identified clinically, e.g., resistance to 3TC and protease inhibitors (Gu 1992).

High levels of cross-resistance do not generally exist among nucleoside analogues. Although, nucleoside analogues are similar structurally, they represent a heterogeneous class of compounds. Not only are different phosphorylation pathways involved in activation, they have different kinetic and inhibition constants, i.e.,

incorporation into elongating DNA. AZT-resistant HIV-1 shows cross resistance only to compounds that contain an azido group. Some cross resistance is seen among ddI, ddC, and 3TC (see below) (Richmann 1991b and 1990b, Wainberg 1993).

Drug-resistant variants of HIV-1, whether selected *in vivo* or *in vitro* has are stable in the absence of drug for several months in tissue culture (Gao,Q. 1992). Over time, there may be a gradual loss of resistance on the part of clinical isolates. It may take up to one year or more for patient isolates to lose resistance after drug discontinuation (Boucher 1993). This suggests that the resistance phenotype does not confer any selective growth advantage in the absence of drug pressure or may be disadvantageous since it is lost over time (Wainberg 1992).

The development of HIV-1 drug-resistance is associated with progression from asymptomatic HIV-1 infection through ARC to AIDS. Resistant viruses are most easily isolated from patients with AIDS, followed by ARC, and least easily from asymptomatic subjects with high CD4 counts (Koziel 1992, Land 1992, Neilsen 1992, Ogino 1993, Richmann 1990a).

Although the emergence of AZT-resistance can be correlated with diminished drug efficacy and disease progression (Ogino 1993), it may not be possible to establish a causative relationship. This is because each of drug resistance, CD4 decline, development of immunoincompetence, and clinical progression will occur at or around the same time.

The mechanisms that underlie drug-resistance are poorly understood. Sequencing of nucleoside analogue-resistant viruses has

revealed a number of mutations in the reverse transcriptase (*pol*) gene. Infectious clones of HIV-1 that contain these mutations have been generated by *in vitro* mutagenesis. Cloning and infection studies with these viruses have confirmed the significance of these mutations, since, the recombinant mutated viruses had IC_{50} values much higher than those of wild type viruses. However, when recombinant RT proteins are generated that contain these mutations, they do not always differ from wild-type proteins in regard to the inhibition constant (K_i) values for ddNTPs (Dianzani 1992, Wood 1987).

Of the many mutations identified in the RT of clinical isolates by cloning, only five have been confirmed by site directed mutagenesis: Thr**215**->Tyr or Phe; Lys**219**->Glu or Gln; Lys**70**->Arg; Asp**67**->Asn or Ser; and Met**41**->Leu. None of the AZT-resistant viruses reported to date contain all five of these mutations. Most do, however, contain a combination of these mutations. There may be a differential and perhaps ordered pattern of acquisition of AZT-resistance-conferring mutation acquisition that exists or that other mutations (Sheehy 1993). With acquisition of additional mutations, the level of AZT-resistance increases. The first mutation to arise is at codon 70 at which stage the virus is still AZT-sensitive. Next, codon 70 reverts to wild type and codon 215 becomes mutated. The 215 mutation persists and mutations at codons 41, 67, and 70 may then appear. Finally, codon 219 may become mutated as well (Richmann1991a). By the time three or four mutations are present, high-level resistance to AZT is usually manifest. The same patterns are seen in patients on prolonged AZT-monotherapy, although the

mutations at codons 67 and 219 have been more difficult to document clinically (Boucher 1992).

In a prolonged AZT-therapy study (longer than 2 years), 11 major mutations in RT from 8 strains of HIV-1 were identified. Thus, other mutations besides the five mentioned above may also account for AZT-resistance (Muckenthaler 1992). These mutations have been mapped to the fingers and the thumb subdomains of HIV-1 RT (D'Aquila 1994).

Mutations thought to confer resistance to ddI include; Lys**65**->Arg, Leu**74**->Val, and Met**184**->Val. Mutations that are thought to confer resistance to ddC include Lys**65**->Arg, and Met**184**->Val, while 3TC-resistance mutations are Lys**65**->Arg and Met**184**->Val (Eron 1993, Fitzgibbon 1992, Gu 1992, Hitchcock 1993, Larder 1993, St Clair 1991, Tisdale 1993, Wainberg 1993).

Drug-resistance may be exerted via different mechanisms for different drugs. IC_{50} values for AZT-resistant viruses can be as high as 100 fold higher than wild-type, whereas, for ddI, IC_{50} values are usually elevated by only 2 to 5 fold (Hitchcock 1993). Perhaps, ddI-resistant viruses have an altered orientation of the terminus primer, such that greater differentiation exists between ddI and the natural dNTP (Eron 1993).

Mutations responsible for nucleoside analogue resistance are spread throughout the HIV-1 RT secondary structure and are far removed from the proposed dNTP binding site. Therefore, they are not likely to interact directly at the template strand at the primer terminus (Kohlstaedt 1992) or to affect the structure of the template binding site. These mutations may alter the enzyme's ability to

discriminate among nucleoside analogues by affecting template positioning (Smerdon 1994).

The goal of this work is to study the effect that AZT has on HIV-1 infection. The methods used for this purpose include tissue culture infection studies, PCR amplification studies, and a re-constituted *in vitro* reverse transcription assay. Specifically, the work will set out to confirm prior observations that AZT preferentially terminates HIV-1 nucleic acid elongation after the first template switch rather than at the production of (-) strong-stop DNA (Arts 1994c). Next, it will attempt to show that nucleoside analogues are more effective chain terminators when deoxynucleoside triphosphate pools are limited. Finally, it will demonstrate that pretreatment of cells with AZT prior to infection has a stimulatory effect on the generation of viral DNA in AZT-resistant variants of HIV-1 but not in wild type or 3TC-resistant variants.

Materials and Methods

A. Cell Culture

MT-4 cells were provided by Dr. N. Yamamoto, Yamaguchi University, School of Medicine, Ube, Japan. The CD4+ Jurkat cell line was obtained from the NIH AIDS Research and Reagent program (Bethesda, Md., U.S.A.). Peripheral blood mononuclear cells (PBMC) were obtained from umbilical cord blood (kindly provided by the Jewish General Hospital's Department of Obstetrics and Gynecology) by density gradient centrifugation (Ficoll-Paque, Pharmacia, Piscataway, NJ). In experiments that utilized quiescent PBMCs, the cells were used immediately. In experiments that required activated PBMCs, Ficoll-Paque isolated PBMCs were stimulated with 10 µg phytohaemagglutinin (Difco, Detroit, Mich.) per ml in supplemented RPMI 1640 medium (see below) for 72 hours prior to subsequent maintenance in 10 U recombinant interleukin-2 (Boehringer-Mannheim, Montreal, Canada). All cell lines were maintained in suspension culture at a concentration of 3 x 10^5 to 5 X 10^5 cells/ml in RPMI-1640 medium (GIBCO Laboratories, Mississauga, Ontario) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Flow Laboratories, Toronto, Ontario, Canada), 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. PBMC's were maintained in the same suspension culture medium supplemented with 5% IL-2 (Boehringer Mannheim, Montreal, Q.C.). All cell lines were maintained in water-jacketed incubators at 37'C and under 5% CO_2. Cells were

mycoplasma-negative, as assessed by fluorescence microscopy using the dye 4',6-diamine-2-phenylindole (DAPI).

B. Virus Culture

The HIV-III_B laboratory strain was a gift of Dr. R.C. Gallo, NIH, Bethesda, MD. The infectious clone, HXB2-D, was acquired from the AIDS Research and Reference Reagent Repository (Rockville, Maryland. The K65R/M184V, K65R, and M184V resistant viral clones were established in our laboratory by site-directed mutagenesis of HXB2-D by Dr. Z. Gu (Gu 1992 and 1994). Clinical isolates (1241, 1245, 1246, 1261, 1266, 1393, 1425) were obtained by coculture of cord blood lymphocytes with peripheral blood lymphocytes from an infected patient as described previously (Rooke 1989). Viral isolates that were recovered were propagated on MT-4 cells as described (Gao,Q. 1992). All viral isolates were maintained at -70'C until use (Gu 1992). Prior to use, virus stocks were filtered and treated with RNase-free DNase I (1 µg/ml; Canadian Life Technologies) for 30 minutes at room temperature in the presence of 10 mM $MgCl_2$ to remove contaminating HIV-1 DNA arising from the lysis of infected cells during preparation (Zack 1992).

C. Drugs

3'-azido-3'-deoxythymidine (AZT) and AZT-TP were obtained from Burroughs-Wellcome Inc. (Research Triangle Park, N.C.). 2',3'-dideoxyinosine (ddI) and ddI-TP were obtained from Bristol-Myers-

Squibb Inc. (Wallingford, CT). 2',3'-dideoxycytidine (ddC) was purchased from Sigma Chemicals Corp. (St. Louis MO). The (-) enantiomer of BCH-189 (3TC) and 3TC-TP were obtained from Glaxo Group Research (Greenford, UK). ddATP, ddITP, and ddCTP were purchased from Sigma (St. Louis, MO). Stock solutions of these drugs were prepared in medium RPMI 1640 and stored at -20'C until use. In experiments that required pretreatment, drug was added to cells 4 hours prior to infection with HIV-1 and maintained until DNA was harvested. Triphosphorylation of drugs to their active forms in cultured cells has been previously described (Furman 1986, Gao,W-Y. 1993b).

D. Assays of Viral Replication

Clarified supernatants were frozen at -70'C until assayed. Measurement of HIV-1 activity in cell-culture supernatant by RT assay was performed as described in the ACTG Virology Manual. Briefly, 50 µL of clarified culture supernatant from test cell cultures was added to 50 µL of reaction cocktail containing: 50 mM Tris hydrochloride (pH 7.9), 5 mM magnesium chloride, 150 mM potassium chloride, 0.5 mM ethylene glycol-bis (b-aminoethyl ether)-N,N,Ní,N-tetraacetic acid, 0.5% Triton X-100, 2% ethylene glycol, 5 mM dithiothreitol, 0.3 mM reduced glutathione, 20 mCi tritiated thymidine triphosphate, and 50 mg/ml of template primer [poly(rA)oligo(dT)] in polypropylene tubes. The tubes were agitated and then incubated at 30'C for 22 hours. The reaction was stopped by the addition of 1 mL of cold 10% (w/v) trichloroacetic acid (TCA).

Newly synthesized DNA was precipitated on ice for at least two hours, and then collected on Whatman GF/C glass fiber filters (Gelman Sciences, Ann Arbor, Michigan) and rinsed twice with cold 10% TCA, and once with absolute ethanol. Filters were dried for 20 minutes and counted in a Packard liquid scintillation analyzer for incorporated radioactivity.

Detection of p24 antigen was by Enzyme-Linked Immunoadsorption Assay (ELISA) in cell-free culture supernatants by using eagents and instructions in the ELISA diagnostic kit (Abbott Laboratories, Toronto, Ontario, Canada).

Stock virus titers ($TCID_{50}$) were quantified using the infectivity assay described in the "AIDS Clinical Trials Group Virus resistance Working Group Consensus Protocol on HIV-1 Drug Susceptibility Testing (for Zidovudine)" version 1.2, 3/18/92. Briefly, 10 fold dilutions of the viral sample were added to wells of a 96-well plate containing 4 x 10^5 PBMC or MT-4/well at a final volume of 250 µl/well. The plate was incubated at 37'C for 4 days. On the fourth day 100 µl of supernatant was recovered and replaced with fresh media. On the seventh day 100 µL of supernatant was recovered from each well for visual inspection for the presence of syncytium, and for determination of reverse transcriptase or p24 levels (described above). The infectious titer was derived from the amount of p24 antigen present and the formul outlined in the protocol.

E. Drug Susceptibility Assays

MT-4 cells were incubated with viruses at a ratio of 0.01 RT units/cell in 1 ml of supernatant. After 2 hours at 37'C, cultures were washed and resuspended. The cell suspensions were plated into 24-well plates containing serial dilutions of the drugs in a volume of 1.5 ml at a concentration of 3 x 10^5 cells/ml. Virus production was assayed 7 days post infection by measuring RT activity in clarified culture supernatants of infected MT-4 cells. IC_{50} values were obtained by curve-fitting analysis.

F. Infections

Cells were either preincubated with drug for 4 hours or not as required in the specific infections. Cells were then infected in 1 ml of DNase I-treated HIV-1 containing supernatants at a ratio of 0.01 RT units/cell in 1 ml of supernatant for either 2 hours (PBMC) or 1 hour (Jurkat or MT-4). Cells were then washed twice with RPMI 1640 complete medium to eliminate unadsorbed virus and incubated for either 12, or 24 hours, or 7 days as indicated in the individual experiments. In those experiments where incubation was for 7 days, half the media was removed and replaced with fresh medium and drug (as required) after four days. Cells were then washed twice in ice-cold phosphate buffered saline, pelleted, and stored at -70'C for lysis and subsequent DNA isolation. Samples of viral culture supernatants were frozen for assays of viral replication (see above). In all infection studies, contamination controls were included that contained no drug and no virus. These controls were carried through to the PCR amplifications. All cellular studies were performed with

DNaseI-treated to minimize levels of viral DNA in viral particles; our methods result in the presence of such DNA at $<10^{-6}$ of that found in infected PBMC (Eric/me ref 3).

G. DNA Isolation and Purification

Low molecular weight (LMW) DNA was isolated by a modification of the HIRT technique (Hirt 1967). One ml of HIRT lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM EDTA, pH 8.0, 0.6% sodium dodecyl sulfate) was used to lyse 1.5 x 10^6 cells. Following precipitation of high-molecular-weight DNA and cellular proteins by the addition of 1 M NaCl, the LMW DNA was obtained by centrifugation of the lysate and ethanol precipitation of the supernatant. The DNA pellet was then resuspended in 50 µl of doubly distilled, de ionized water.

For whole cell DNA extractions, cells were lysed in TNE (10 mM Tris-Cl, pH 7.6, 100 mM KCl, and 10 mM EDTA, pH 8.0)-0.4% SDS buffer. The cells and lysis buffer were incubated overnight at 37'C with and 20 mg/mL pronase. Samples were subjected to two phenol chloroform extractions. The DNA was precipitated by addition of 2.5 volumes of 95% ethanol and 1/10 volume ammonium acetate at -20'C overnight. The DNA pellet was resuspended in distilled water. The concentration of DNA was quantitated by spectrophotometry.

H. PCR Amplifications

All reagents used for nucleic acid preparation were specifically prepared for polymerase chain reaction (PCR) use and tested to ensure that no HIV-1 DNA contamination was present.

The PCR cocktail (100 µl) contained 5 µl (1/5 of the total) HIRT LMW DNA or 1 µg whole cell DNA, 100 pmol of each primer (labelled sense and unlabelled antisense) (described below) and consisted of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 2 U Taq polymerase (Canadian Life Technologies), 0.2 mM dATP, 0.2 uM dGTP, 0.2 uM dCTP, 0.2 uM dTTP (Pharmacia Laboratories) and 50 pmol each of sense and anti-sense oligonucleotide primers. Samples were overlaid with 100 µL of light mineral oil and then subjected to thermal cycling in a Perkin-Elmer Cetus Thermal Cycler.

The deoxyoligonucleotide primers used in the amplifications were designed from the HXB2-D genome and modeled after those previously described (Zack 1990). Refer to Figure 4A for a full description of the primers. The general locations of the primer pairs are represented schematically in Fig. 4B (Zack 1992). The primer pair A13 and S1 amplifies a 140 bp segment in the U5 region of the LTR that corresponds to (-) strong stop DNA. The primer pair A2 and S2 amplifies a 207 bp segment in the U3 region of the LTR that corresponds to first template switch DNA. The primer pair AG4 and SG4 amplifies a 95 bp segment upstream of the *gag* gene that corresponds to near full length HIV-1 DNA. The primer pair Ga and Gs amplifies a 110 bp segment in the cellular β-globin gene and was used as an internal control to normalize the amount of DNA loaded into PCR reactions as previously described (Zack 1990). The primer

pair MTA2 and MTS2 amplifies a 130 bp segment in the non-coding region of mitochondrial DNA and was also used as an internal control to normalize the amount of DNA loaded into PCR reactions (as previously described) (Anderson 1981). The amplification efficiencies of the various primer pairs varied according to the strength of primer annealing (A13/S1 > A2/S2 > AG4/SG4). Thus estimates of PCR-amplified product were made in comparison with known copy numbers of *Xho*-linearized HXB2-D HIV-1 plasmid. It should be noted that there are two copies of A13/S1 and A2/S2 segments but only one copy of the AG4/SG4 segment in fully transcribed HIV-1 proviral DNA. Also, a linearized copy of HXB2-D contains only one AG4/SG4 amplification site.

Sense primers were end-labelled via an end labeling exchange reaction using [32(P]-ATP (1 mCi/400 pmol of primer) (Amersham, Oakville, Canada) and added with cold antisense primers to the DNA samples. Serial dilutions (1/10) of *XhoI*-linearized HXB2-D (Gelezuinas AAC 1994 ref 31), which contains a full-length HIV-1 genome, were used to establish a standard control for PCR amplification in order to establish the template copy number. Samples were subjected to 27 cycles of denaturation for 1 min at 94 'C, annealing for 1.5 min at 60 'C, and polymerization for 1 min at 72 'C. PCR products were run on 8% denaturing polyacrylamide gels, which were then dried, autoradiographed, and subjected to GS-250 Molecular Imager (BioRad) analysis.

I. In Vitro Reverse Transcription Reactions

The RNA template, HIV RNA PBS template was produced by in vitro transcription reactions from the AccI-linearized pHIV-PBS plasmid using T7 RNA polymerase as described previously (Arts 1994b) (see Figure 5). The HIV-1 RNA PBS template (0.5 pmol) was then added to a reaction cocktail (20 µl) containing 10 mM DTT, 50 mM Tris-HCl, pH 7.8, 100 mM KCl, 10 mM $MgCl_2$, 1, 10 or 100 µM of each of the four deoxynucleoside -5'-triphosphates (dNTP) as indicated, and no drug or ddITP, ddCTP, ddATP, 3TC-TP, or AZT-TP at either 1/2 or 1/10 the dNTP concentration, as indicated. The reactions also contained 2.5 pmol of either human $tRNA^{Lys,3}$ or 18 nucleotide (nt) deoxyoligonucleotide complimentary to the PBS (termed dPR), and (α^{32}P)-dCTP and (α^{32}P)-dATP (Amersham) at 1/20 the concentration of dNTP.

Reverse transcription cocktails were denatured at 85 'C for 2 min, cooled to 55 'C for 8 min, and further cooled to 37 'C prior to the addition of HIV-1 RT at 42,5 nM (kindly provided by Dr. L. Martin, Welcome Inc., N.C.) and incubation for 1 hour at 37 'C. A double stranded DNA sequencing reaction was also performed by Dr. Eric Arts on the AccI-linearized pHIV-PBS (0.05 pmol) with the (γ^{32}P)-labelled dPR primer using a Taq sequencing reaction kit (Canadian Life Technologies, Burlington, Ontario, Canada). This sequencing reaction provided a marker to determine the sites of ddNTP chain termination. The reaction products were purified as described previously (Arts 1995 and 1994a), then run on a 5% denaturing polyacrylamide gel which was then dried and analyzed by a GS-250 Bio-Rad molecular imager.

*This experiment was performed in conjunction with Dr. Eric Arts.

Results

A. Preferential chain termination after the first template switch by AZT in cases of infection with wild type HIV-1

PBMC were pretreated with 0.1 or 1 μ*M* AZT for 4 hours prior to exposure to HIV-1 HXB2-D at an M.O.I. of 0.01 RT units/cell. The infection was allowed to continue for 24 hours after which time, whole cell DNA was isolated. This was subjected to PCR amplification to determine levels of (-) strong-stop DNA, post first template switch DNA, and near full length DNA present in the different samples. As shown in Figure 6A, there was considerable amplification of (-) strong-stop HIV-1 DNA in all samples (lane 3, no drug, 6 x 10^6 copies; lane 2, 0.1 μ*M* AZT, 3 x 10^6 copies, lane 1, 1 μ*M* AZT, 1 x 10^6 copies). These results confirm earlier findings (Arts 1994c, Gelezuinas 1993) that (-) strong-stop DNA produced prior to template switching was made even in the presence of nucleoside analogues. In contrast, levels of post first template switch and near full length DNA were diminished in the presence of AZT (lanes 1 and 2, Figure 6 B and C) relative to the absence of AZT (lane 3, Figure 6 B and C). Figure 7 graphs the percentage of HIV-1 DNA in the 0.1 and 1 mM AZT samples (Figure 6 A, B, and C, lanes 1 and 2) relative to the level of each species of HIV-1 DNA in the no drug controls (Figure 6 A, B, and C, lanes 3). These ratios indicate relative extent of HIV-1 reverse transcription in the generation of (-) strong-stop, post first template switch, and near full length HIV-1 DNA in the presence of nucleoside analogues.

B. Synthesis of (-) strong stop DNA in HIV-1-infected, nucleoside analogue-pretreated quiescent and PHA-stimulated PBMC

The LMW DNA of HIV-1-infected, nucleoside analogue-pretreated PBMC was isolated by HIRT extraction (Arts 1994c) and subjected to PCR amplification as described in the Materials and Methods. Using the A13/S1 primer pair, HIV-1 (-) strong stop DNA was identified and quantified in untreated and nucleoside analogue-pretreated quiescent- or PHA-stimulated PBMC infected with wild type HIV-1 (Figure 8A). To quantitate differences, the amounts of (-) strong stop DNA PCR amplified products were standardized to an internal control, i.e. amounts of DNA mitochondrial product amplified by the MTA2/MTS2 primer pair (not shown). As a quantitation control, ten-fold serial dilutions of linearized HXB2-D DNA were also PCR amplified with A13/S1 (left-hand portion of Figure 8A). In the absence of nucleoside analogue treatment of PBMC, there was more (-) strong stop DNA amplified by PCR in PHA-stimulated PBMC ($>10^6$ copies) (Figure 8A, lane 1) than in quiescent PBMC ($<10^5$ copies, lane 9). This difference in levels of reverse transcription in quiescent compared to PHA-stimulated PBMC has been previously reported (Dahlberg 1987, Zimmerman 1987).

Levels of products obtained in the case of nucleoside analogue-treated samples (Figure 8A, lanes 2-8 for PHA-stimulated PBMC and lanes 10-16 for quiescent PBMC) were determined by measuring the intensities of relevant bands by phosphor-imaging relative to those of untreated samples (Figure 8A, lanes 1 and 9, respectively). In each

case, an arbitrary value of 1 was assigned to products generated in the absence of drug (Figure 8A, lanes 1 and 9). Standard deviations were calculated on the basis of three independent experiments and are indicated as error bars in Figure 8B. In the case of quiescent PBMC, we observed a significant decrease in levels of PCR-amplified (-) strong stop DNA in cells treated with each nucleoside analogue as compared with untreated cells (Figure 8B). No such significant differences were seen in comparisons of untreated PHA-stimulated PBMC versus PHA-stimulated PBMC treated with 0.01, 0.1 or 1 μM AZT, 10 or 100 μM ddI, or 5 or 50 μM 3TC. These findings in PHA-stimulated PBMC are consistent with previous observations (Arts 1994c). We have obtained similar results using concentrations of AZT between 0.001 - 100 μM (data not shown).

C. Chain termination by ddNTP in cell-free reverse transcription reactions

Cell-free RT assays have shown that AZT-TP is preferentially incorporated into elongating DNA resulting in chain termination after the first template switch in reactions primed by $tRNA^{Lys,3}$ but not dPR (Arts 1994c). Some chain termination also occurs during synthesis of (-) strong-stop DNA in reactions primed by $tRNA^{Lys,3}$, but only with high concentrations of AZT-TP and to a lesser extent with dPR primer (Arts 1994c). This subject was further investigated by monitoring synthesis of (-) strong-stop DNA in reactions primed by either dPR or $tRNA^{Lys,3}$ in the absence or presence of various ddNTP concentrations.

Figure 9A displays the products of RT reactions primed with $tRNA^{Lys,3}$ at 100 μM dNTP. Figure 9B, lanes 1 to 33, shows the products of similar reactions primed with dPR in the presence of three different concentrations of dNTP (i.e. 100 μM, lanes 1-11; 10 μM, lanes 12-22; and 1 μM, lanes 23-33) and ddNTP (at 1/2 and 1/10 the concentration of corresponding dNTP). Lane 1 of figure 9A and lanes 1, 12, and 23 of figure 9B display the products of reactions performed in the absence of drug. Pause sites are indicated by letters in Figure 9A and B. Sites of incorporation of ddNMPs, resulting in chain termination, are visualized as non-pause product bands and are not present in lane 1 in figure 9A and lanes 1, 12, and 23 in Figure 9B.

Reactions containing ddNTP at 1/2 of dNTP concentrations showed more chain termination and less synthesis of (-) strong DNA

than those that contained ddNTP at 1/10 the dNTP concentration. However, considerable variation was observed with regard to levels of chain termination in the presence of different nucleoside analogues. AZT-TP, ddATP, and ddCTP caused more chain termination than did ddITP and 3TC-TP (lanes 8 to 11; Figure 9A and B). These results show that ddITP can be incorporated by HIV-1 RT, resulting in chain termination opposite guanidine residues in the RNA template, in spite of the fact that ddATP is believed to constitute the intracellular active form of ddI (Johnson 1989).

Decreased ddNTP concentrations and the use of dPR in the place of tRNALys,3 caused augmented chain termination by ddNTP (1/2 and 1/10 the concentration of dNTP) and decreased synthesis of (-) strong-stop DNA (Figure 9B). Figure 10 is an analysis of the levels of (-) strong-stop DNA generated in the presence of ddNTP relative to those produced in the absence of drug. Augmented chain termination was observed with all ddNTPs concomitant with use of diminished concentrations of dNTP. Increased chain termination under these conditions was most apparent in reactions performed with the weak chain terminators, i.e. ddITP and 3TC-TP (Figure 10).

*This experiment was performed in conjunction with Dr. Eric Arts.

D. Characterization of AZT-resistant clinical isolates of HIV-1

Clinical isolates of HIV-1 that were obtained by coculture of uninfected PBMC with PBMCs from HIV-1 infected donors who had been undergoing long-term AZT monotherapy were characterized as described in the materials and methods. Table 1 indicates that all clinical isolates (with the exception of isolate 1261 and 1266) showed similar levels of infectivity. The data in this table was used to equalize the level of infectious virus that was used in the infection studies. Table 2 shows that all clinical isolates (with the exception of isolate 1261) showed resistance to AZT. The level of resistance ranged from 72 fold higher IC_{50} values (isolate 1241) relative to wild type to 276 fold higher IC_{50} values (isolate 1245). The average IC_{50} was 143 +/- 76 fold higher than the wild type IC_{50} value. Table 3 shows the results of the genetic analysis of the clinical isolates. The results show that all of the clinical isolates (with the exception of 1241) contain the major AZT-resistance mutation Thr215Tyr. Isolate 1241 has only the 70 mutation and is likely to represent an early population of AZT-resistant virus (Richman 1991a). This is reflected in the isolate's IC_{50} (0.180 μM) which is the lowest of all of the clinical isolates. Isolates 1245, 1266, and 1425 have both the Thr215Tyr and the Met41Leu mutations that are known to confer high level AZT-resistance. These isolates probably represent a more advanced population of AZT-resistant viruses (Richman 1991a). This is reflected in their high levels of AZT-resistance (IC_{50} for 1245 = 0.690 μM, 1266 = 0.380 μM, and 1425 = 0.420 μM). Isolate 1246

contains only the Thr215Tyr mutation and shows intermediate AZT-resistance (IC_{50} = 0.270 μM). Isolate 1393 contains a Thr215Ile and Lys70Arg mutation and also shows an intermediate level of AZT-resistance (IC_{50} = 0.200 μM)

E. Synthesis of (-) strong-stop DNA following infection by AZT-resistant clinical isolates of HIV-1

LMW DNA of HIV-1-infected, nucleoside analogue-pretreated (1 μ*M* AZT) PBMC was isolated by HIRT extraction (Arts 1994c) and subjected to PCR amplification as described in the Materials and Methods. Using the A13/S1 primer pair, HIV-1 (-) strong stop DNA was identified and quantified in untreated and AZT-treated PBMC infected with wild type HIV-1 (Figure 11A). To quantitate differences, the amounts of (-) strong stop DNA PCR amplified products were standardized to an internal control, i.e. amounts of DNA mitochondrial product amplified by the MTA2/MTS2 primer pair. As a quantitation control, ten-fold serial dilutions of linearized HXB2-D DNA were also PCR amplified with A13/S1 (right-hand portion of Figure 11A).

Figure 11 shows that all AZT-resistant isolates showed significantly more (average = 3.4 fold higher) PCR-amplified (-) strong-stop DNA in the presence of 1 μ*M* AZT than there was in the absence of the drug. The stimulatory effect of AZT was not observed in wild type HIV-1 (laboratory strain HIV_{IIIB}). The levels of products obtained were determined by measuring the intensities of the relevant bands by phosphor-imaging analysis. These levels were plotted for each isolate relative to the levels obtained for that particular isolate in the absence of drug treatment. Standard deviations were calculated on the basis of three independent experiments and are indicated as error bars in Figure 12.

F. Generation of ddI resistance in otherwise ddI-sensitive and AZT-resistant clinical isolates of HIV-1

3 x 10^6 PBMCs were treated with either no drug, AZT or ddI, or combinations of AZT and ddI as indicated (Figure 13) for 4 hours prior to infection with wild type (HIV_{IIIB}) or AZT-resistant (1241, 1245, 1393) cultures of HIV-1. Aliquots of the viral supernatants were assayed at 7 days post infection for p24 levels and the values obtained were plotted relative to the no drug samples which were assigned an arbitrary value of 1. There was a decrease observed in p24 levels for wild type HIV-1 in all drug samples. There was a similar decrease in the AZT-resistant isolates in the samples that contained only ddI. The decrease observed in the AZT-resistant samples that contained AZT was consistent with the level of resistance for each virus. p24 was generated in the presence of ddI in AZT-resistant isolates when small amounts (0.0001 to 0.1 μM) AZT were also present. This effect was diminished as the concentration of both ddI and AZT increased. Results for clinical isolates 1245 and 1393 (not shown) were similar to those obtained for clinical isolate 1241.

*This experiment was only performed one time successfully due to technical difficulties and time constraints

G. Characterization of 3TC-resistant infectious clones of HIV-1

Infectious clones of HIV-1 generated by Dr. Z. Gu in our lab were characterized as described in the materials and methods. Table 4 indicates that there was variation in the infectivity level of the different clones. Wild type (HXB2-D) was the most infectious, followed by Δ184 (about half as infectious), then by Δ65 (about 1/4 as infectious), and by Δ184/65 (about 1/3 as infectious). The data in this table was used to equalize the level of infectious virus that was used in the infection studies. Table 5 shows that all of the clones are sensitive to AZT but resistant to 3TC with the double mutant (Δ184/65) showing the highest level of resistance. Table 6 summarizes the sequencing results on the infectious clones and confirms their genetic make-up.

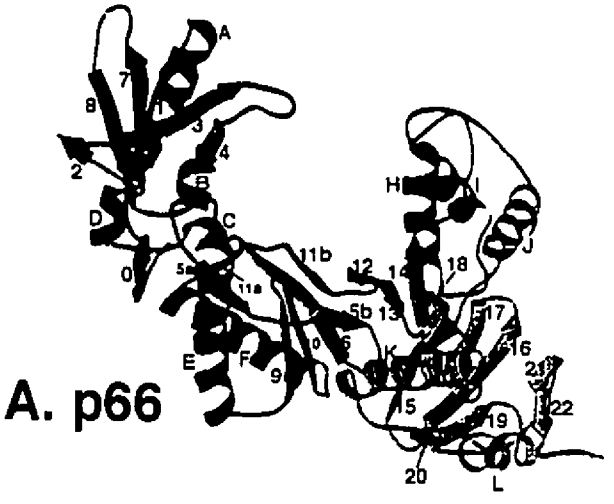
H. Generation of (-) strong-stop DNA by 3TC-resistant infectious clones of HIV-1 in the presence of AZT or 3TC

Nucleoside analogue pre-tretaed PBMC (Figure 14 pretreated with AZT as indicated, Figure 15 pre-treated with 3TC as indicated) were infected with wild type (HXB2-D) or 3TC-resistant clones (Δ65, Δ184, and Δ184/65) of HIV-1 as indicated in the legends to Figure 14 and Figure 15. Either LMW DNA (Figure 14, AZT), or whole cell DNA (Figure 15, 3TC) was isolated as described in the Materials and Methods and subjected to PCR amplification. Using the A13/S1 primer pair, HIV-1 (-) strong stop DNA was identified and quantified in untreated and drug-treated PBMC infected with wild-type or 3TC-resistant infectious clones of HIV-1 (Figure 14, Δ65, Δ184, and HXB2-D; and Figure 15, Δ65, Δ184, Δ184/65, and HXB2-D). To quantitate differences, the amounts of (-) strong stop DNA PCR amplified products were standardized to an internal control, i.e. amounts of low molecular weight mitochondrial DNA product amplified by the MTA2/MTS2 primer pair (Figure 14) and amounts of high molecular weight β-globin product amplified by the GA/GS primer pair (Figure 15). As a quantitation control, ten-fold serial dilutions of linearized HXB2-D DNA were also PCR amplified with A13/S1 (left hand portion of Figure 14 and right hand portion of Figure 15, respectively).

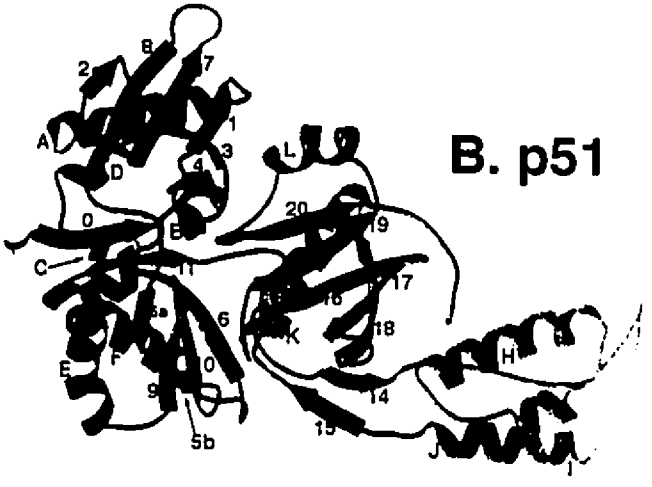
Both Figures 14 and 15 show that there is an equivalent amount of (-) strong-stop DNA product generated in the presence or absence of either AZT or 3TC for both wild type and 3TC-resistant infectious clones of HIV-1

Figure 1. Crystal stucture of the p66 an p51 subunits of HIV-1 reverse transcriptase.

This figure was modified from the 2.4 Å resolution of the crystal structure of HIV-1 RT complexed with dsDNA (Jacob-Molina1993) by Dr. Eric Arts. The β and α greek letters represent the predicted beta sheet and alpha helix secondary structures in the subunits.



A. p66



B. p51

LEGEND

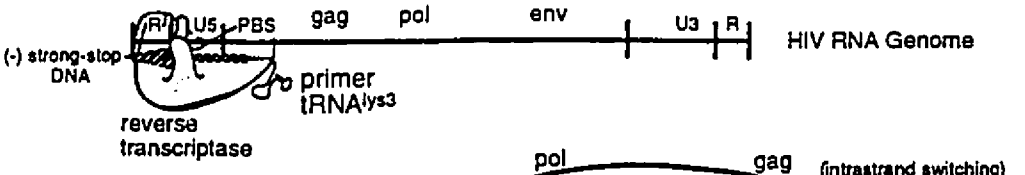
Subdomains of HIV-1 reverse transcriptase

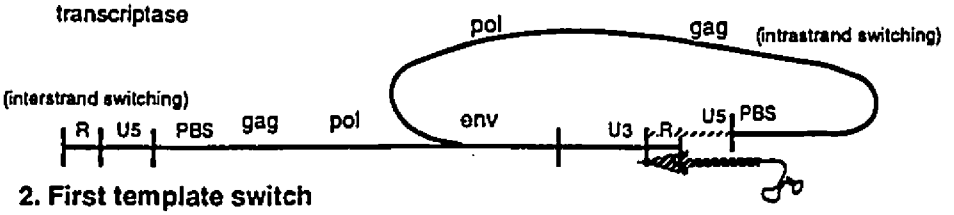
- Fingers
- Palm
- Thumb
- Connection
- polymerase active site
- dNTP binding site

a.a. sequence	Secondary structure
7-24	β0-β1
28-44	αA
49-77	β2-β4
78-83	αB
86-112	β5a-β6
114-127	αC-αD
128-147	β7-β8
155-174	αE
178-191	β9-β10
195-212	αF
214-242	β11a-β14
255-311	αH-αJ
316-358	β15-β18
364-382	αK
388-391	β19
395-404	αL
408-430	β20-β22

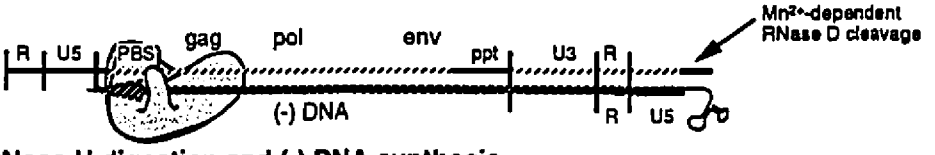
Figure 2. Scheme of HIV-1 reverse transcription.
This figure is a schematic representation of the process of HIV-1 reverse transcription. It was adapted from a figure that was initially designed by Dr. Eric Arts. For details about the particular steps in the process, refer to Section D (Reverse Transcription) in the text above.

1. Initiation of RDDP

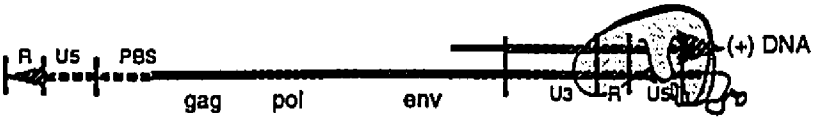




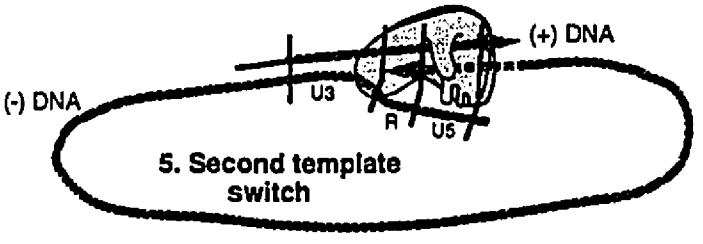
2. First template switch



3. RNase H digestion and (-) DNA synthesis



4. ppt priming of DDDP



5. Second template switch

LEGEND

	single-stranded DNA
	DNA synthesized due to interstrand switching
	single-stranded RNA
	RNase H digested RNA
RDDP	RNA-dependent DNA polymerization
DDDP	DNA-dependent DNA polymerization
ppt	polypurine tract

6. Completion of ds proviral DNA

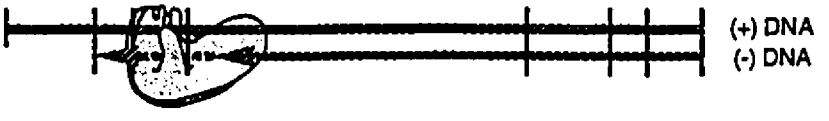


Figure 3. Scheme of nucleoside analogue phosphorylation. This figure is a schematic representation of the intracellular phosphorylation by cellular enzymes of AZT and ddI to their active triphosphate forms. For details about the particular steps in the process, refer to Section E (Treatment Strategies) in the text above.


AZT
thymidine kinase
AZT-MP
thymidylate kinase
AZT-DP
pyrimidine nucleoside diphosphatase
AZT-TP

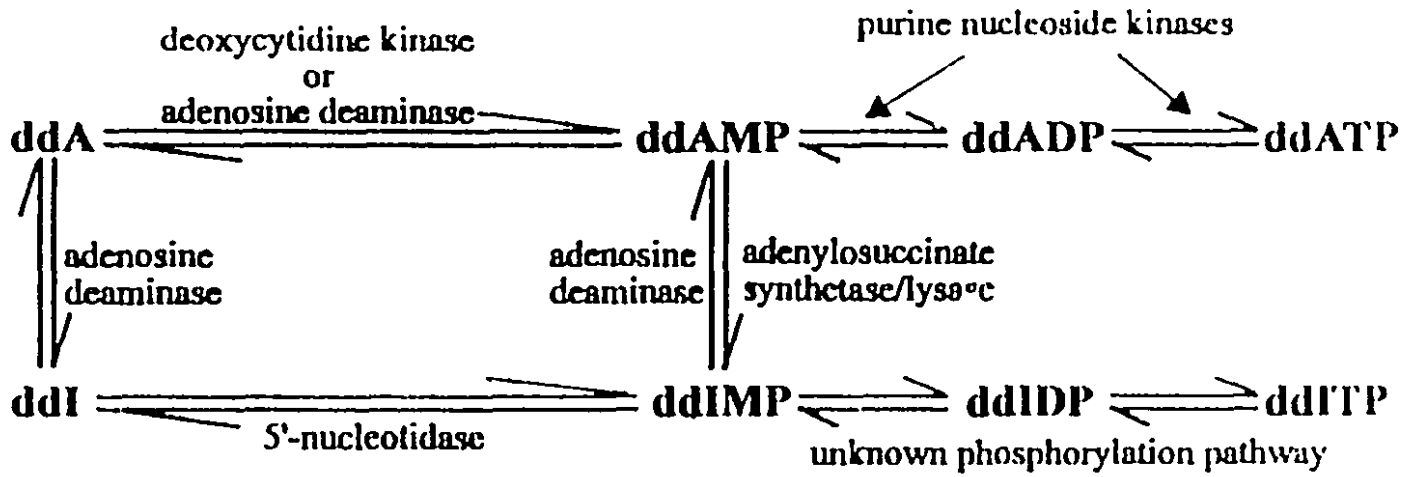
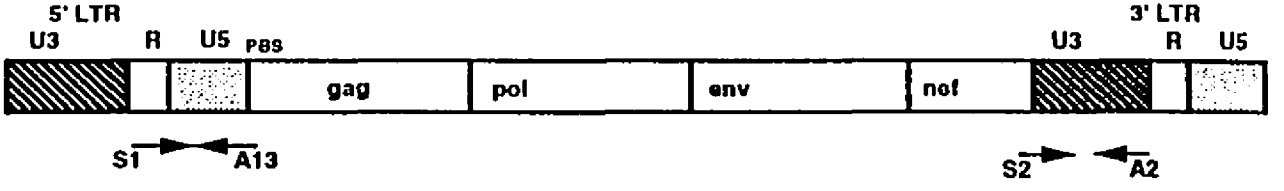

deoxycytidine kinase
or
adenosine deaminase
ddA
ddAMP
purine nucleoside kinases
ddADP
ddATP
adenosine deaminase
adenosine deaminase
adenylosuccinate synthetase/lyase
ddI
ddIMP
ddIDP
ddITP
5'-nucleotidase
unknown phosphorylation pathway

Figure 4. PCR primers.

This figure is a schematic representation of the HIV-1 genome and the primer pairs that were used in PCR experiments to amplify different spiecies of the genome. Primer pair A13/S1 were used to amplify a segment of the HIV-1 genome corresponding to (-) strong-stop DNA. Primer pair A2/S2 were used to amplify a segment of the HIV-1 genome corresponding to post-template switch DNA. Primer pair AG4/SG4 were used to amplify a segment of the HIV-1 genome corresponding to near full length DNA. For further details, refer to Materials and Methods, Section H (PCR Amplifications) in the text above.

A. HIV PROVIRAL DNA



B. PRIMERS

C. REPLICATION SCHEME

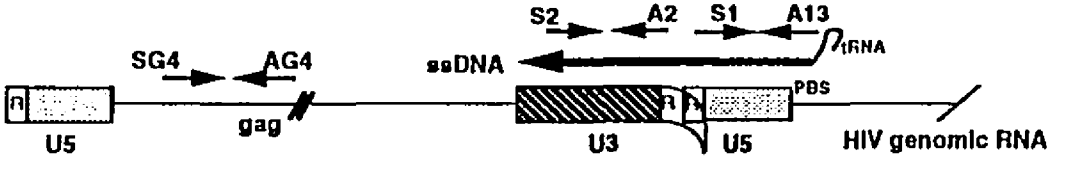


Figure 5. Schematic of *in vitro* reverse transcription reaction.

This figure is a schematic representation of the *in vitro* reverse transcription reactions that were carried out (in conjunction with Dr. Eric Arts) in the absence or presence of different concentrations of dNTPs and nucleoside analogues (ddNTPs) with either tRNA(Lys,3) or a deoxynucleotide (dPR) primer. For further details, refer to Materials and Methods, Section I (*In Vitro* Reverse Transcription Reactions) in the text above.

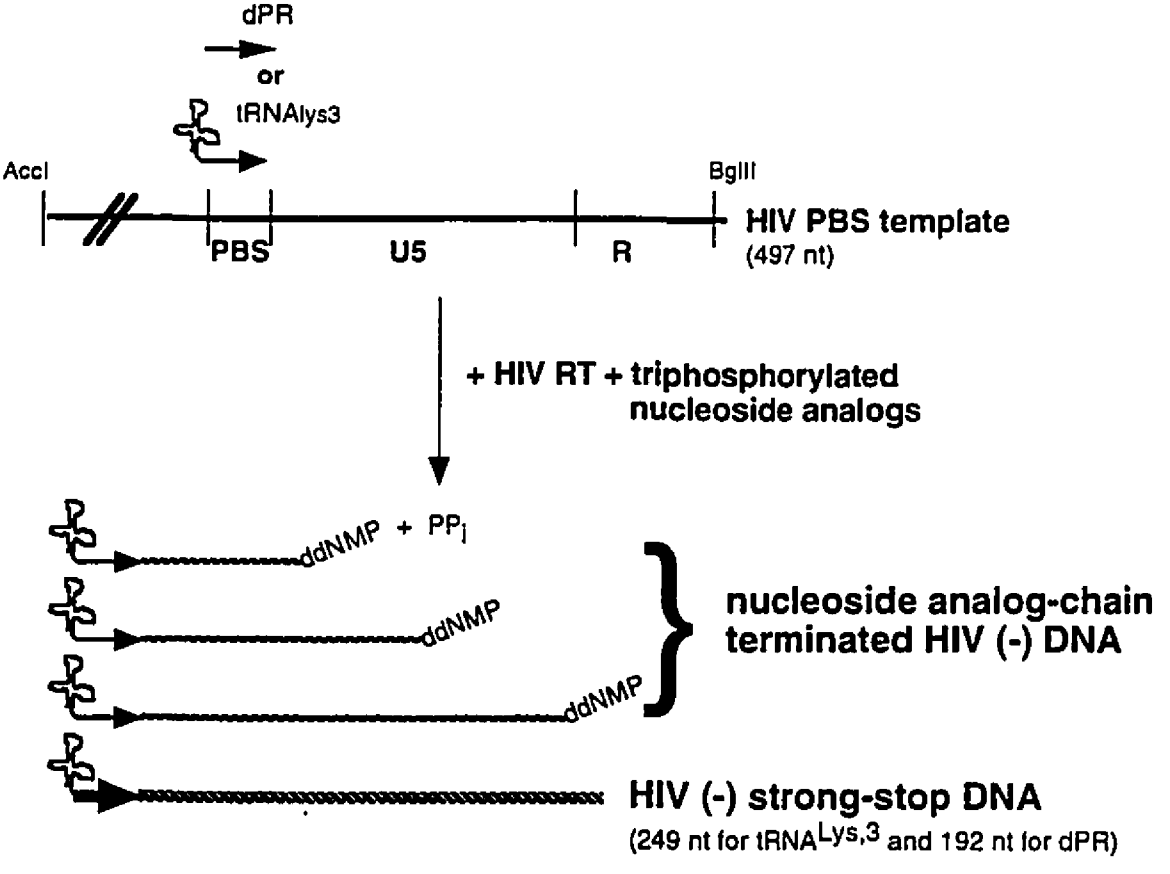

dPR
or
tRNAlys3
AccI
BglII
PBS
U5
R
HIV PBS template
(497 nt)
+ HIV RT + triphosphorylated nucleoside analogs
ddNMP + PPi
ddNMP
ddNMP
nucleoside analog-chain terminated HIV (-) DNA
HIV (-) strong-stop DNA
(249 nt for tRNALys,3 and 192 nt for dPR)

Figure 6. PCR amplification of (-) strong-stop, post-first template switch, and near full-length HIV-1 DNA in HIV-1 infected, AZT-pretreated PBMC.

Whole cell DNA was obtained from AZT pre-treated PBMC 24 hours post infection with wild type (HXB2-D) HIV-1 as described in Materials and Methods. 1 μg of this DNA and 10-fold serial dilutions of *Xho*I-linearized HXB2-D were then subjected to 27 cycles of PCR amplification with three different sets of HIV-1 specific primer pairs. Panel A depicts a 140 bp fragment of HIV-1 amplified by the A13/S1 primer pair that corresponds to (-) strong stop DNA. Panel B depicts a 207 bp fragment of HIV-1 amplified by the A2/S2 primer pair that corresponds to post-template switch (-) DNA. Panel C depicts a 95 bp fragment of HIV-1 amplified by the AG4/SG4 primer pair that corresponds to a near full-length (-) DNA. The products were electrophoresed on a 7% denaturing polyacrylamide gel which was dried, autoradiographed and subjected to quantitation analysis with a BioRad GS-250 Molecular Imager. β-globin DNA was amplified (data not shown) as described in Materials and Methods as an internal control to guarantee that observed differences were not due to differences in the amounts of input DNA into the PCR reactions.

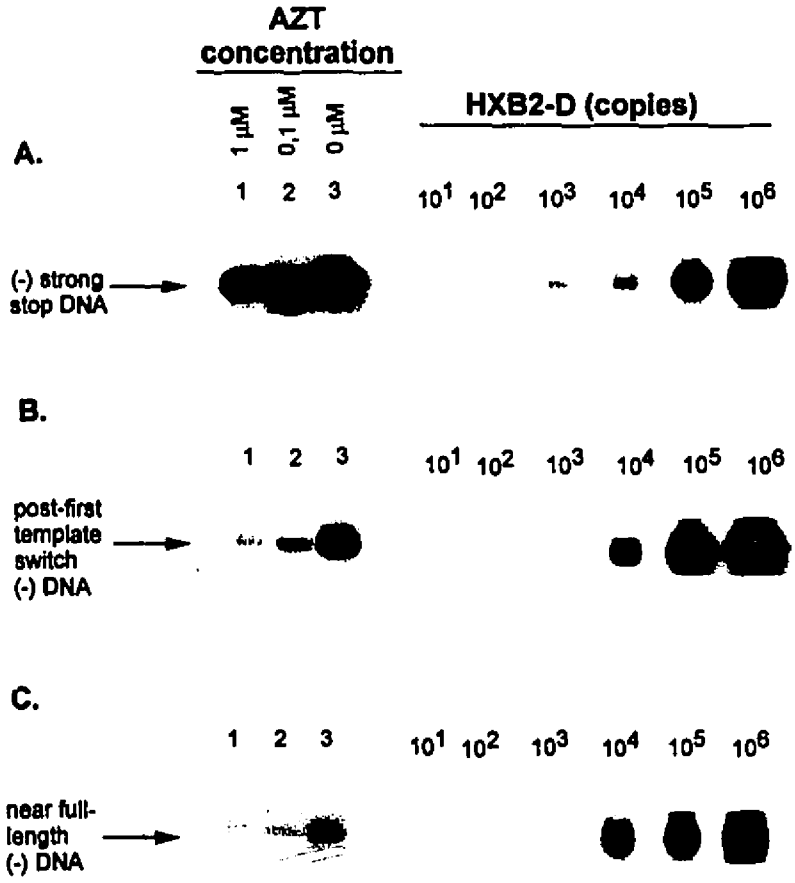

AZT concentration
1 µM
0,1 µM
0 µM
HXB2-D (copies)
A.
1 2 3
10^1 10^2 10^3 10^4 10^5 10^6
(-) strong stop DNA
B.
1 2 3
10^1 10^2 10^3 10^4 10^5 10^6
post-first template switch (-) DNA
C.
1 2 3
10^1 10^2 10^3 10^4 10^5 10^6
near full-length (-) DNA

Figure 7. Graphic analysis of PCR amplification of (-) strong-stop, post-first template switch, and neafull-length HIV-1 DNA from HIV-1 infected, AZT-pretreated PBMC. The percentages of each respective species of HIV-1 DNA are plotted relative to the no drug control sample for each of (-) strong-stop DNA, post-first template switch DNA, and near full length DNA.

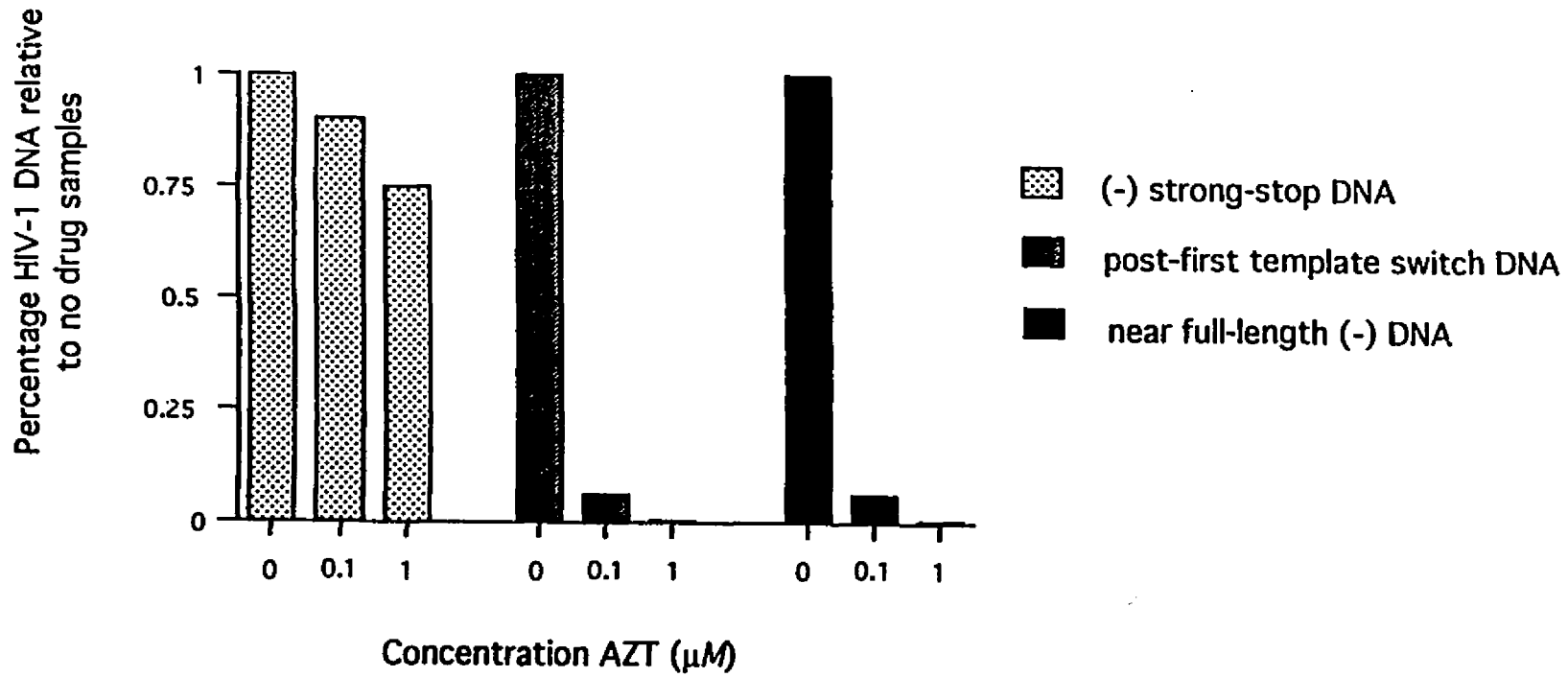
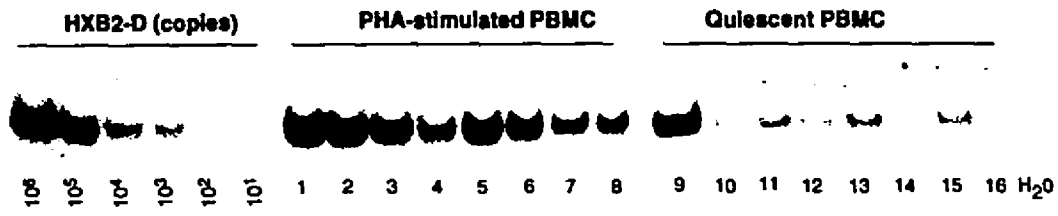

Percentage HIV-1 DNA relative to no drug samples
1
0.75
0.5
0.25
0
0
0.1
1
0
0.1
1
0
0.1
1
Concentration AZT (μM)
(-) strong-stop DNA
post-first template switch DNA
near full-length (-) DNA

Figure 8. PCR amplifications of LMW DNA from HIV-1-infected, nucleoside analogue-pretreated quiescent and PHA-stimulated PBMC.

Low molecular weight extracts from quiescent and PHA-stimulated PBMC that had been treated with no drug, AZT (0.01, 0.1 or 1 μM), ddI (10 or 100 μM), or 3TC (5 or 50 μM) were PCR amplified using the A13/S1 primer pair which amplifies a 140 bp fragment of (-) strong stop DNA in the HIV-1 genome). Ten fold dilutions from 10^6 to 10^1 copies of *Xho*I-linearized HXB2-D plasmid were PCR amplified with the A13/S1 primer pair as a positive quantitation control. Panel A shows the products of these reactions on an 8% denaturing polyacrylamide gel (Lanes 1 and 9, no drug; lanes 2 and 10, AZT 0.01 μM; lanes 3 and 11, AZT 0.1 μM; lanes 4 and 12, AZT 1 μM, lanes 5 and 13, ddI 10 μM; lanes 6 and 14 ddI 100 μM; lanes 7 and 15, 3TC 5 μM; and lanes 8 and 16, 3TC 50 μM). A mock reaction which included only water was included as a PCR control. The intensity of each product was determined using a BioRAD 250 GS Molecular Imager and the results were plotted in panel B. Amounts of (-) strong stop DNA in quiescent and PHA-activated nucleoside analogue-treated PBMC were adjusted relative to those of untreated infections of PBMC, given arbitrary values of 1. Mitochondrial DNA was amplified (data not shown) as described in Materials and Methods as an internal control to guarantee that differences were not due to different amounts of input DNA into the PCR reactions.

A.


HXB2-D (copies)
PHA-stimulated PBMC
Quiescent PBMC
10⁶ 10⁵ 10⁴ 10³ 10² 10¹ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 H2O

B.

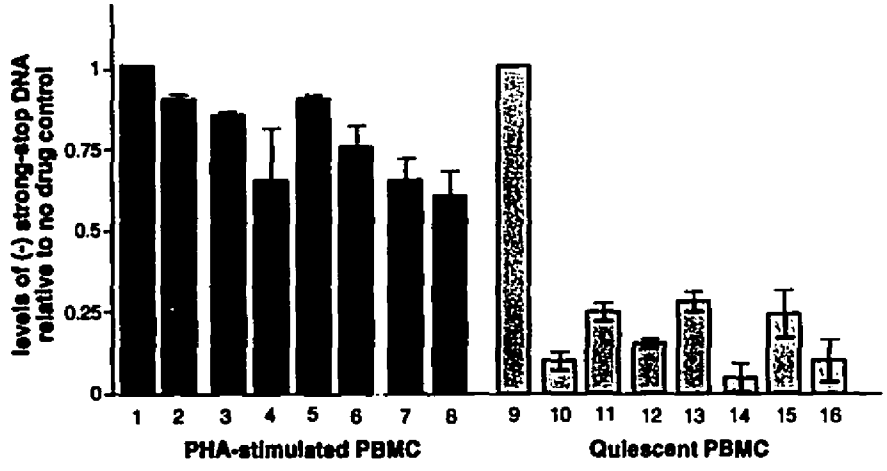

levels of (-) strong-stop DNA relative to no drug control
1
0.75
0.5
0.25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
PHA-stimulated PBMC
Quiescent PBMC

Figure 9. The effects of altering dNTP concentrations and of the presence or absence of nucleoside analogue-triphosphates on HIV-1 reverse transcriptase pausing and chain termination with a dPR or $tRNA^{lys,3}$ primer on a natural RNA template.

In vitro reverse transcription reactions were carried out using wild type recombinant reverse transcriptase as described in Materials and Methods. Panel A shows results obtained using a natural $tRNA^{lys,3}$ primer in the presence of 100 μM of each of the four dNTPs and either the absence or presence of different nucleoside analogue triphosphates as indicated. The concentration of nucleoside analogue utilized in each lane is indicated as 1/2 or 1/10 of the concentration of the indicated dNTP concentration. Panel B shows the results obtained using a natural dPR primer in the presence of either 100 μM , 10 μM , or 1 μM of each of the four dNTPs and either the absence or presence of different nucleoside analogue triphosphates as indicated. (-) ss represents full length (-) strong stop DNA and letters A through N represent major pause sites on the RNA template that was utilized in the *in vitro* reverse transcription reaction.

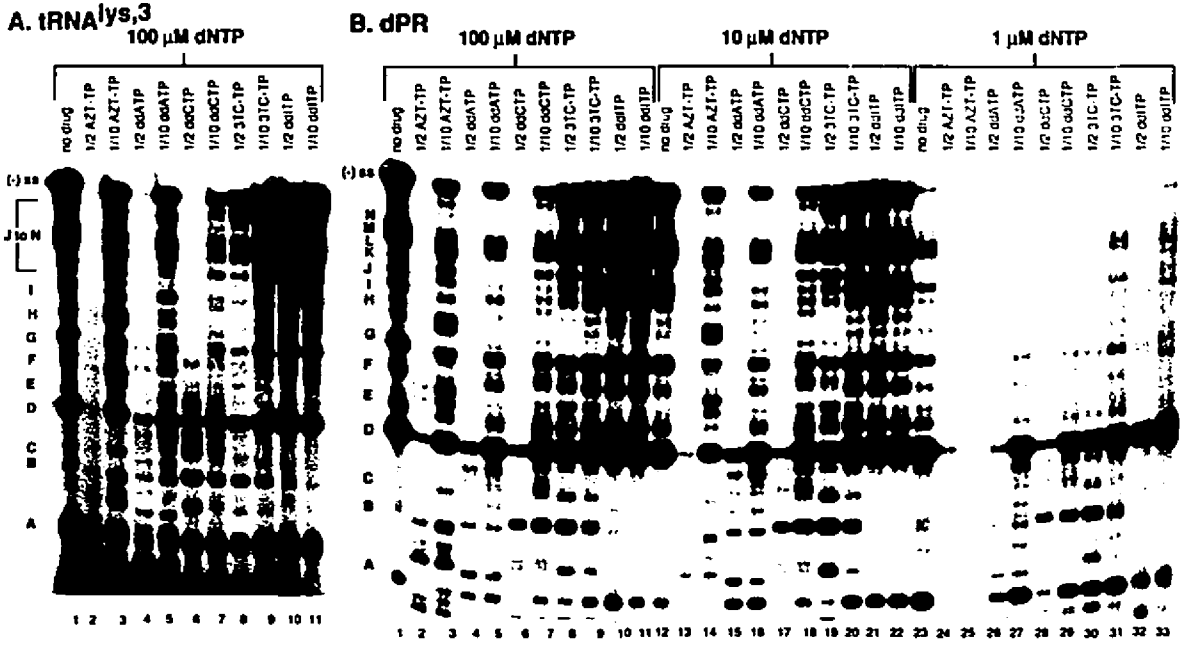

A. tRNA^lys,3
100 µM dNTP
B. dPR
100 µM dNTP
10 µM dNTP
1 µM dNTP
no drug
1/2 AZT-TP
1/10 AZT-TP
1/2 ddATP
1/10 ddATP
1/2 ddCTP
1/10 ddCTP
1/2 3TC-TP
1/10 3TC-TP
1/2 ddITP
1/10 ddITP

Figure 10. Graphical analysis of theeffects of altering dNTP concentrations and of the presence orabsence of nucleoside analogue-triphosphates on HIV-1 reverse transcriptase pausing and chain termination with a dPR or a $tRNA^{lys,3}$ primer on a natural RNA template.

The amounts of (-) strong-stop DNA shown in Figure 4 were determined by phosphorimaging analysis and are plotted in Figure 5. Amounts of (-) strong-stop DNA product in the case of untreated samples were arbitrarily assigned values of 1 for reactions primed with $tRNA^{Lys,3}$ (+ or - 100 μM dNTP), and dPR at varying dNTP concentrations (+ or - 100 μM, 10 μM, or 1 μM dNTP).

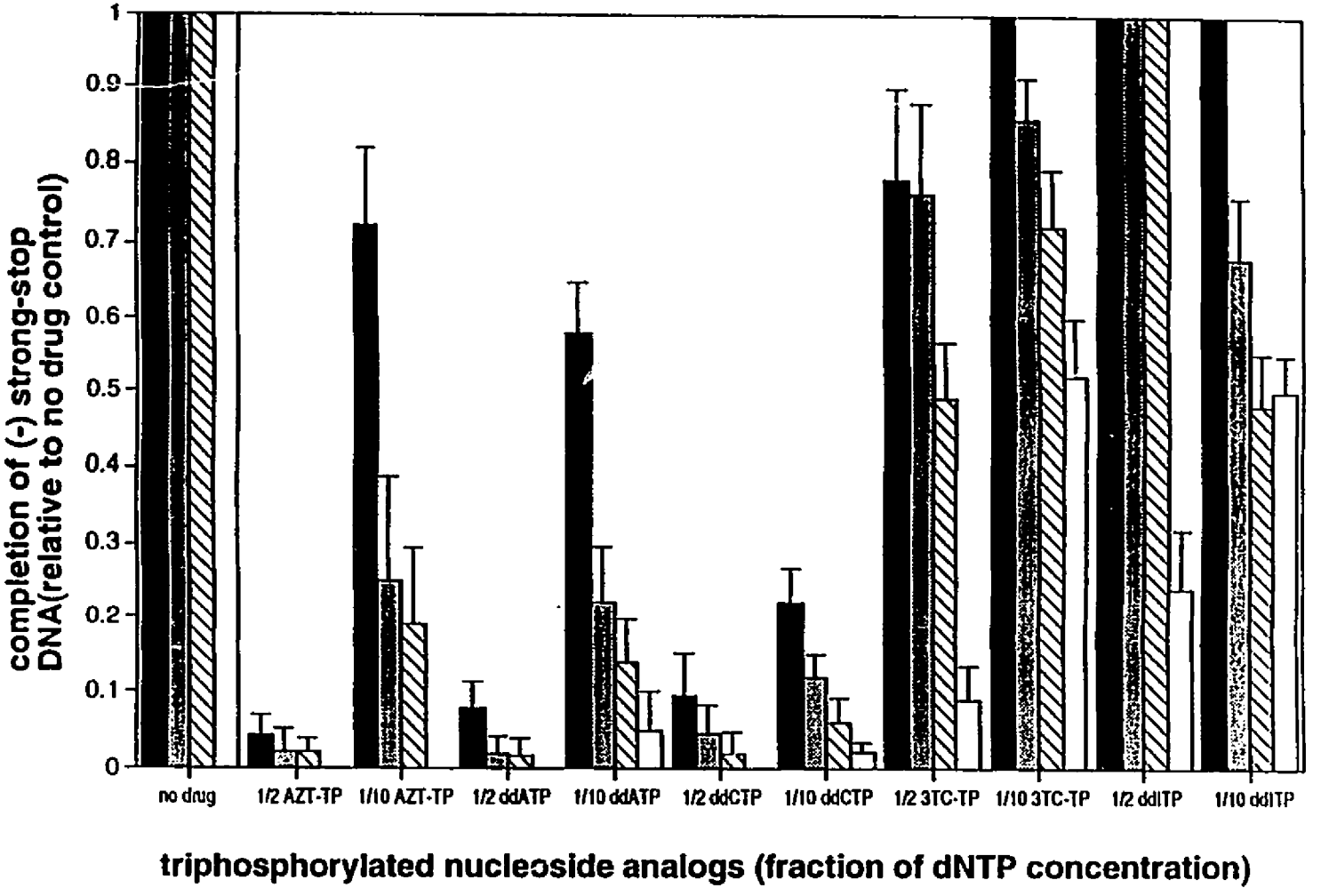

completion of (-) strong-stop DNA(relative to no drug control)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
no drug
1/2 AZT-TP
1/10 AZT-TP
1/2 ddATP
1/10 ddATP
1/2 ddCTP
1/10 ddCTP
1/2 3TC-TP
1/10 3TC-TP
1/2 ddITP
1/10 ddITP
triphosphorylated nucleoside analogs (fraction of dNTP concentration)

Figure 11. Generation of (-) strong stop HIV-1 DNA in the presence or absence of AZT in wild type vs AZT-resistant clinical isolates of HIV-1.

PCR amplifications of whole cell DNA obtained from PBMC infected with either wild type (WT) or AZT-resistant (1241, 1245, 1246, 1266, 1393, 1425) clinical isolates of HIV-1 in the absence (-) or presence (+) of 1 μM of AZT were conducted as described in Materials and Methods utilizing the A13/S1 primer pair that amplifies a 140 bp fragment of HIV-1 DNA that corresponds to (-) strong stop DNA and the MTA2/MTS2 primer pair that amplifies a 130 bp fragment of human mitochondrial DNA as an internal quantitation control. Serial dilutions of 10^2 to 10^6 copies of linearized HXB2-D plasmid were also PCR-amplified as described in Materials and Methods and used as positive quantitation controls to determine the copy numbers of HIV-1 DNA species generated in the infection study.

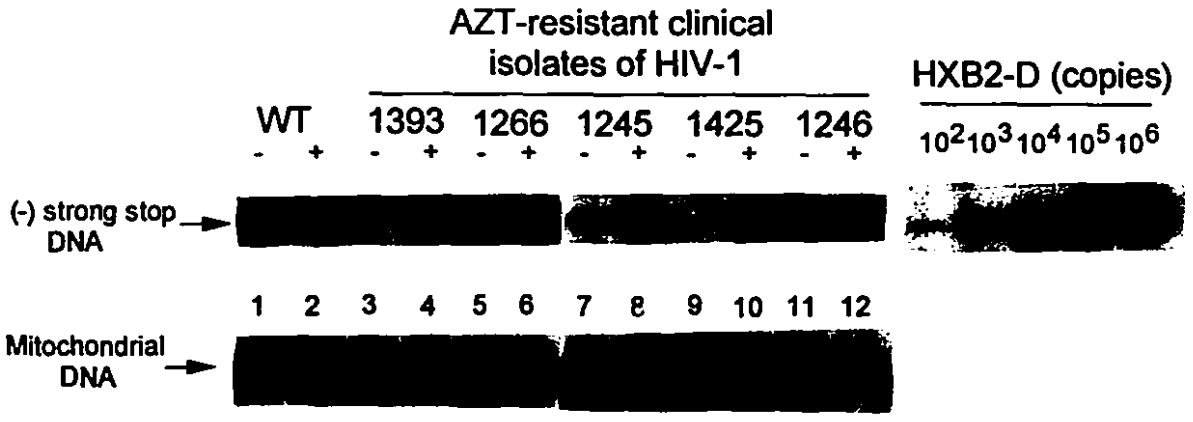

AZT-resistant clinical isolates of HIV-1
HXB2-D (copies)
WT
1393
1266
1245
1425
1246
- + - + - + - + - + - +
10^2 10^3 10^4 10^5 10^6
(-) strong stop DNA
1 2 3 4 5 6 7 8 9 10 11 12
Mitochondrial DNA

Figure 12. Graphical analysis ofthe generation of (-) strong stop HIV-1 DNA in the presence or absence of AZT in wild type vs AZT-resistant clinical isolates of HIV-1 in PBMCs. The levels of (-) strong-stop DNA in the presence of 1 μM AZT are plotted relative to the levels of (-) strong-stop DNA in the absence of AZT (assigned a value of 1) for each HIV-1 clinical isolate studied of HIV-1. Each experiment was performed in triplicate. The error bars indicate standard deviation in three separate identical experiments.

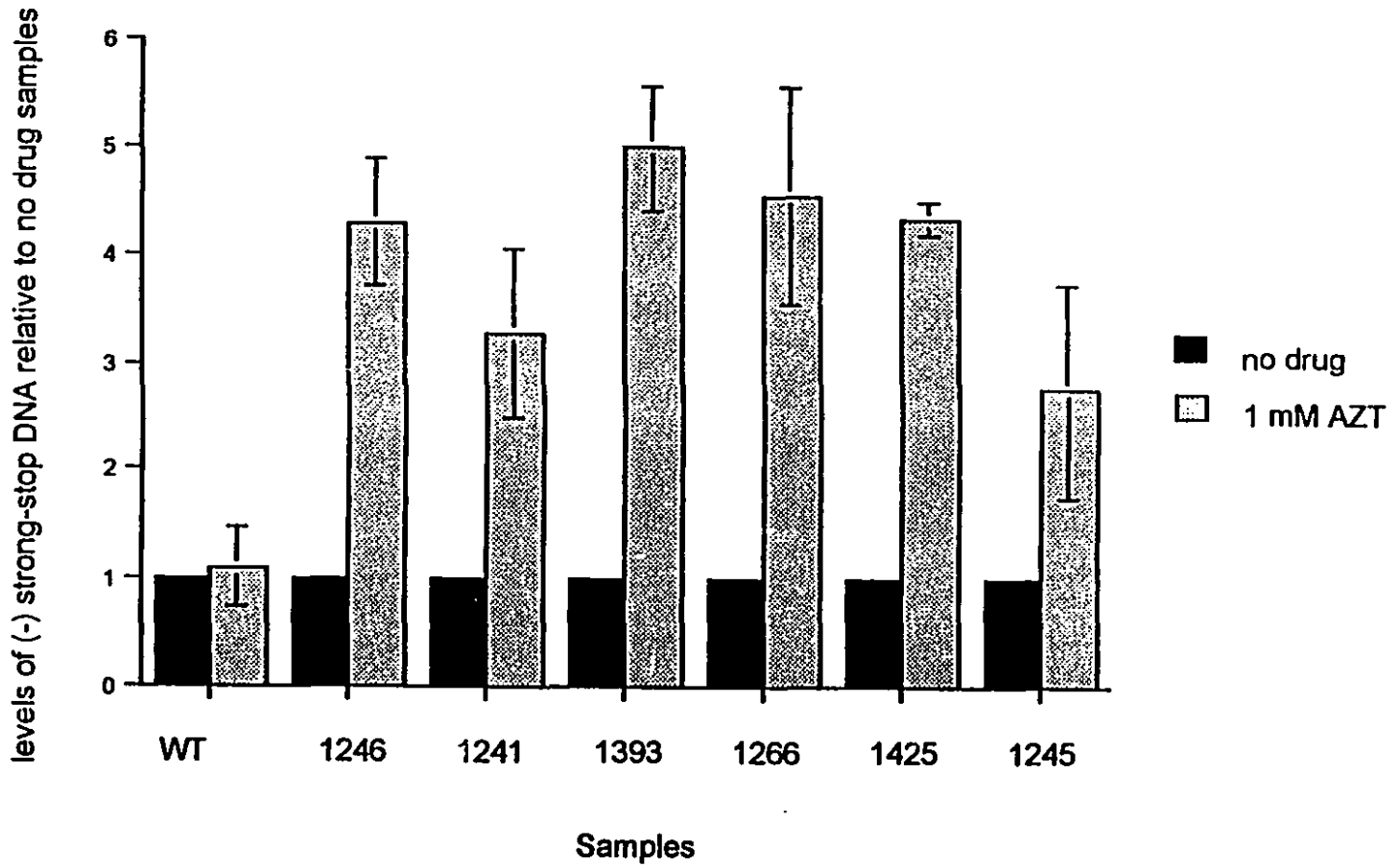
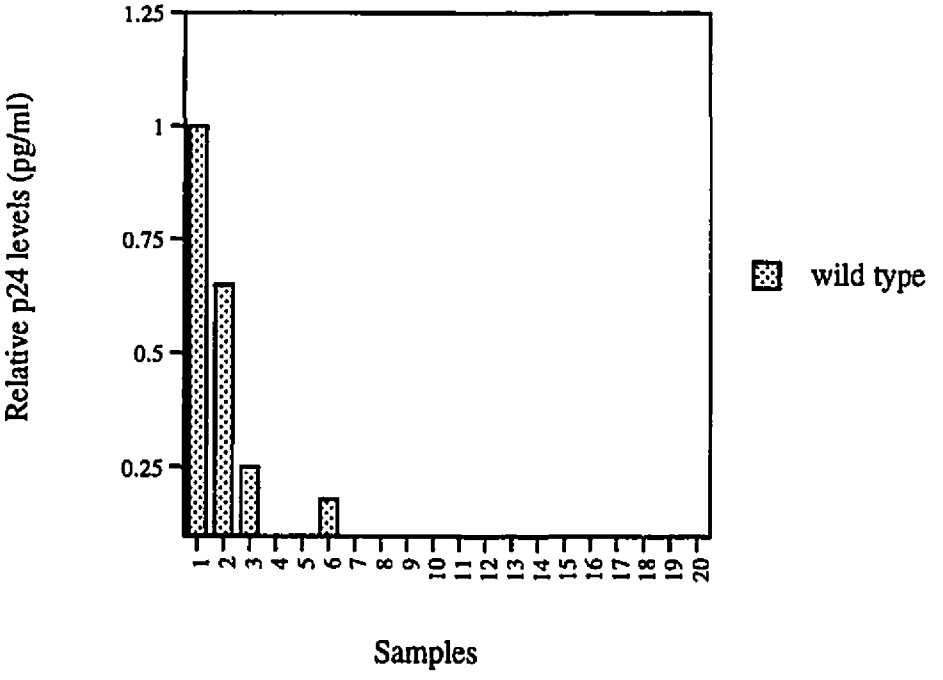

levels of (-) strong-stop DNA relative to no drug samples
0
1
2
3
4
5
6
WT
1246
1241
1393
1266
1425
1245
Samples
no drug
1 mM AZT

Figure 13. Generation of ddI-resistance to otherwise ddI-sensitive and AZT-resistant HIV-1 by infecting in the presence of low concentrations of AZT.

Infection of Jurkat cells with wild type or AZT-resistant HIV-1 (clinical isolate 1241) was carried out as described in Materials and Methods in the presence or absence of drug as indicated; (1) no drug, (2) 0.001 μ*M* AZT, (3) 0.01 μ*M* AZT, (4) 0.1 μ*M* AZT, (5) 1 μ*M* AZT, (6) 0.5 μ*M* ddI, (7) 5 μ*M* ddI, (8) 50 μ*M* ddI, (9) 0.5 μ*M* ddI and 0.001 μ*M* AZT (10) 5 μ*M* ddI and 0.001 μ*M* AZT (11) 50 μ*M* ddI and 0.001 μ*M* AZT, (12) 0.5 μ*M* ddI and 0.001 μ*M* AZT, (13) 5 μ*M* ddI and 0.01 μ*M* AZT (14) 50 μ*M* ddI and 0.01 μ*M* AZT, (15) 0.5 μ*M* ddI and 0.1 μ*M* AZT, (16) 5 μ*M* ddI and 0.1 μ*M* AZT (17) 50 μ*M* ddI and 0.1 μ*M* AZT, (18) 0.5 μ*M* ddI and 1 μ*M* AZT, (19) 5 μ*M* ddI and 1 μ*M* AZT (20) 50 μ*M* ddI and 1 μ*M* AZT.

Wild Type Virus (IC_{50} (AZT) = 0.0025 μM)



Clinical Isolate 1241 (IC_{50} (AZT) = 0.400 μM)

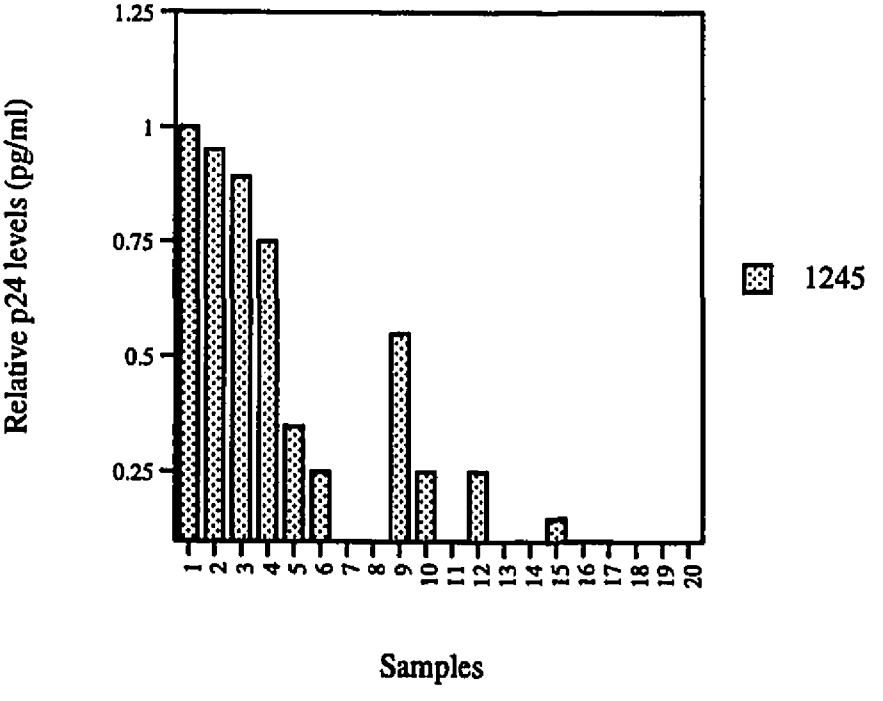


Figure 14. Generation of (-) strong stop HIV-1 DNA in the presence or absence of AZT in wild type vs 3TC-resistant (Δ 65, and Δ 184) infectious clones of HIV-1.

PCR amplifications of LMW DNA obtained from PBMC infected with either wild type (HXB2-D) or 3TC-resistant (Δ 65, and Δ 184) infectious clones of HIV-1 in the absence or presence of AZT were conducted as described in Materials and Methods utilizing the A13/S1 primer pair that amplifies a 140 bp fragment of HIV-1 DNA that corresponds to (-) strong stop DNA and the MTA2/MTS2 primer pair that amplifies a 130 bp fragment of human mitochondrial DNA as an internal quantitation control. The products of these reactions are shown on an 8% denaturing polyacrylamide gel (Lanes 1, 5, and 9, no drug; Lanes 2, 6, and 10, 0.001 μM AZT; Lanes 3, 7, and 11, 0.01 μM AZT; and Lanes 4, 8, and 12, 0.1 μM AZT). Serial dilutions of 10^1 to 10^6 copies of linearized HXB2-D plasmid were also PCR-amplified as described in Materials and Methods and used as positive quantitation controls to determine the copy numbers of HIV-1 DNA species generated in the infection study.

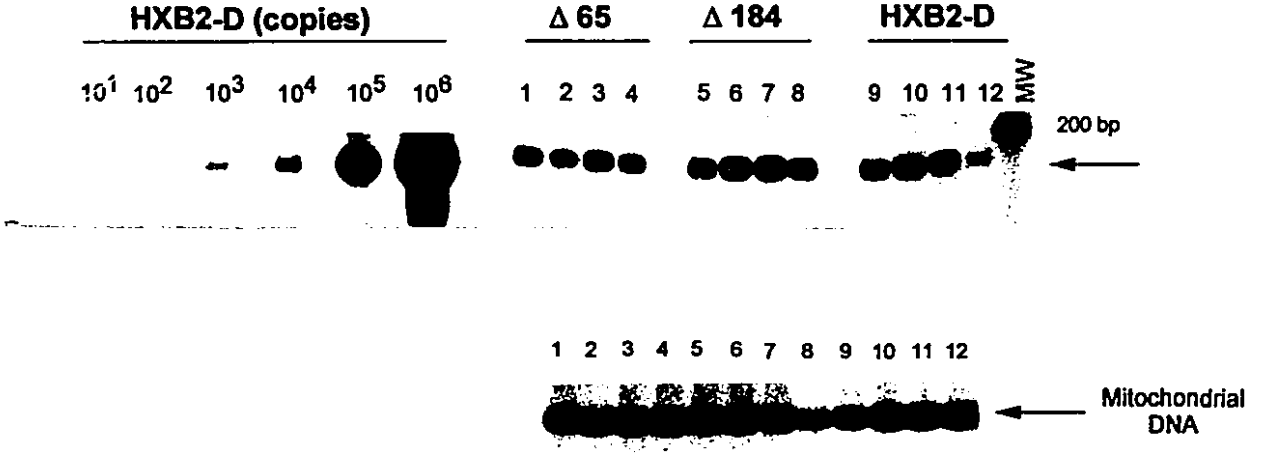
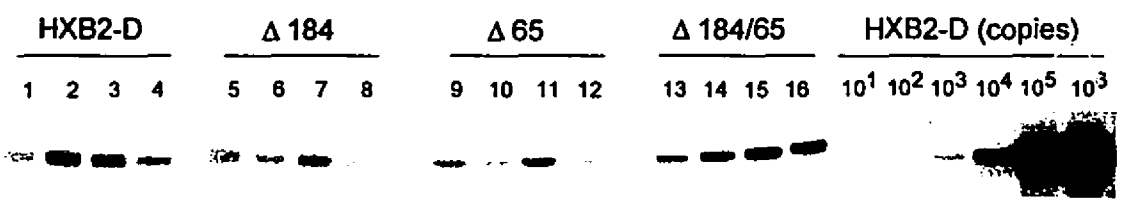

HXB2-D (copies)
Δ 65
Δ 184
HXB2-D
10^1 10^2 10^3 10^4 10^5 10^6
1 2 3 4
5 6 7 8
9 10 11 12
MW
200 bp
1 2 3 4 5 6 7 8 9 10 11 12
Mitochondrial DNA

Figure 15. Generation of (-) strong stop HIV-1 DNA in the presence or absence of 3TC in wild type vs 3TC-resistant (Δ 65, Δ 184, and Δ 184/65) infectious clones of HIV-1.

PCR amplifications of whole cell DNA obtained from PBMC infected with either wild type (HXB2-D) or 3TC-resistant (Δ **65,** Δ **184,** and Δ **184/65)** infectious clones of HIV-1 in the absence or presence of 3TC were conducted as described in Materials and Methods utilizing the A13/S1 primer pair that amplifies a 140 bp fragment of HIV-1 DNA that corresponds to (-) strong stop DNA and the GA/GS primer pair that amplifies a 110 bp segment in the cellular β-globin gene as an internal quantitation control. The products of these reactions are shown on an 8% denaturing polyacrylamide gel (Lanes 1, 5, 9 and 13, no drug; Lanes 2, 6, 10 and 14, 0.01 μM 3TC; Lanes 3, 7, 11, and 15, 1 μM 3TC; and Lanes 4, 8, 12, and 16, 100 μM 3TC). Serial dilutions of 10^1 to 10^6 copies of linearized HXB2-D plasmid were also PCR-amplified as described in Materials and Methods and used as positive quantitation controls to determine the copy numbers of HIV-1 DNA species generated in the infection study.


HXB2-D
Δ 184
Δ 65
Δ 184/65
HXB2-D (copies)
1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16
10^1 10^2 10^3 10^4 10^5 10^3

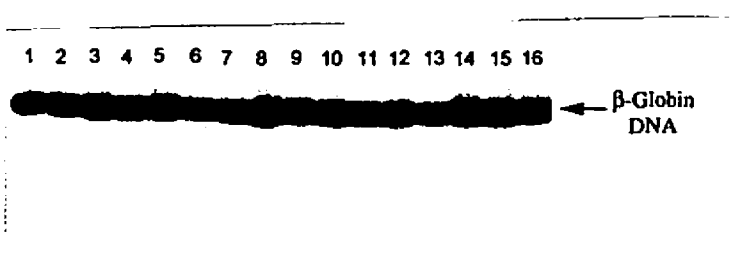

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
β-Globin
DNA

Table 1. Infectiousness data re clinical isolates of HIV-1[a]

HIV-1 Isolate	p24 (pg/ml)[b] 1/100	1/1000	1/5000	RT[c] (cpm)	7 days p.i RT[d] (cpm)	TCID50[e] (units)
wild type	>414	205	32,0	803 000	1 100 000	465 000
1241	>414	260	43,6	580 000	1 300 000	656 000
1245	>414	220	40,6	507 000	1 200 000	656 000
1246	>414	248	42,1	270 000	700 000	365 000
1261	32	ND[f]	ND	69 000	10 000	ND
1266	309	45	ND	170 000	500 000	235 000
1393	>414	210	41,6	377 000	1 000 000	656 000
1425	>414	195	32,0	219 000	700 000	465 000

a data represents the means of 3 replicate samples.

b,c p24 antigen capture and reverse transcriptase assays were performed directly on frozen stocks of HIV-1.

d MT-4 cells were infected with the indicated virus and 7 days post infection culture supernatants were tested for reverse transcriptase activity.

e serial 10 fold dilutions of virus was used to infect MT-4 cells and 7 days post infection, cultures were scored as either (+) or (-) for the presence of virally-induced CPE and tissue culture infectivity dose values were determined according to the formula described in the AIDS Clinical Trials Group Consensus Protocol on HIV-1 Drug Susceptibility Testing (Version 3/18/92)

f ND indicates not done

Table 2. Sensitivity data re clinical isolates of HIV-1 to AZT[a]

HIV-1 isolate	IC50 (μM AZT)	fold increase over wild type
wild type	0.0025	1
1241	0,180	72
1245	0,690	276
1246	0,270	108
1261	ND[b]	ND
1266	0,380	152
1393	0,200	80
1425	0.420	168

a Virus prouction was assessed by the measurement of reverse transcriptase activity in clarified supernatants of infected MT-4 cells 7 days post-infection. IC50 values were then determined by curve-fitting analysis.

b ND indicates not done

Table 3. Mutational analysis of nucleoside analogue-resistant clinical isolates of HIV-1

Isolate	IC50 (μM)[a] AZT	TCID50[b] (x 10e6 units)	Mutations[c] 41	70	215	219	other
wild type	0.0025	0.465	Met	Lys	Thr	Lys	-
1241	0.180	0.656	-	Arg	-	-	75,134
1245	0.690	0.656	Leu	-	Tyr	-	64
1246	0.270	0.365	-	-	Tyr	-	50,135,180
1261	ND[d]	ND	-	-	Tyr	-	50,80
1266	0.380	0.235	Leu	-	Tyr	-	-
1393	0.200	0.656	-	Arg	Ile	-	90,125,13
1425	0.420	0.465	Leu	-	Tyr	-	39,158

a IC50 values are from table 3

b TCID50 values are from table 1

c The identification of all of these mutants was carried out by Dr. Z.Gu by sequencing analysis; (-) indicates wild type codon; (amino acid) indicates mutant codon

d ND indicates not done

Table 4. Infectiousness data re recombinant clones of HIV-1 *a*

HIV-1 isolate	p24 (pg/ml)*b*			RT*c*	7 days p.i RT*d*	TCID50*e*
	1/100	1/1000	1/5000	(cpm)	(cpm)	(units)
HXB2-D	>414	261	54,0	2 195 389	3 830 900	3 303 923
Δ 65	>414	96	20,0	548 564	716 281	520 032
Δ 184	>414	237	49,0	1 430 937	1 607 900	654 681
Δ 65/184	>414	72	13,0	437 609	103 205	257 649

a data represents the means of 3 replicate samples.

b,c p24 antigen capture and reverse transcriptase assays were performed directly on frozen stocks of HIV-1.

d MT-4 cells were infected with the indicated virus and 7 days post infection culture supernatants were tested for reverse transcriptase activity.

e serial 10 fold dilutions of virus were used to infect MT-4 cells and 7 days post infection, cultures were scored as either (+) or (-) for the presence of virally-induced CPE and tissue culture infectivity dose values were determined according to the formula described in the AIDS Clinical Trials Group Consensus Protocol on HIV-1 Drug Susceptibility Testing (Version 3/18/92)

Table 5.Sensitivity data re recombinant clones of HIV-1 infectious clones to AZTand 3TC*a*

HIV-1 isolate	IC50 (μM)	
	AZT	3TC
HXB2-D	0.0007 (1 X)	0.025 (1 X)
Δ 65	0.0009 (1,3 X)	0.400 (16 X)
Δ 184	0.0015 (2,1 X)	25.0 (1000 X)
Δ 65/184	0.007 (10 X)	>100 (>40000X)

a Virus prouction was assessed by the measurement of reverse transcriptase activity in clarified supernatants of infected MT-4 cells 7 days post-infection. IC50 values were then determined by curve-fitting analysis.

c parentheses indicate level of resistance above wild types

Table 6. Mutational analysis of nucleoside analogue-resistant infectious clones of HIV-1*a*

Isolate	IC50 (μM)*b*		TCID50*c*	Mutations*d*						
	AZT	3TC	(x 10e6units)	41	65	67	70	184	215	219
HXB2-D	0.0007	0.025	ND	Met	Lys	Asp	Lys	Met	Thr	Lys
Δ 65	0.0009	0.400	ND	-	Arg	-	-	-	-	-
Δ 184	0.0015	25.0	ND	-	-	-	-	Val	-	-
Δ 65/184	0.007	>100	ND	-	Arg	-	-	Val	-	-

a Infectious clones were generated by Dr. Z. Gu (ref)
b IC50 values are from table 7
c TCID50 values are from table 6
d (-) indicates wild type codon; (amino acid) indicates mutant codon. These mutations were confirmed by sequencing analysis

Discussion

The effectiveness of nucleoside analogues has been undermined by the rapid ability of HIV-1 to mutate and develop high level resistance to drugs that are targeted against it. To circumvent this problem, a better understanding of the mechanisms involved is required.

This work has three major themes. First, we used infection studies and PCR to confirm that AZT can cause preferential chain termination following the first template switch. Second, we showed that nucleoside analogues are more effective chain terminators when dNTP pools are limiting (e.g., in quiescent PBMC) than when they are not (e.g., PHA-activated PBMC). Third, we have demonstrated that AZT may enhance reverse transcription and the generation of viral DNA in the case of AZT-resistant clinical isolates of HIV-1.

A common denominator in these studies is the processivity of the RT of HIV-1 . Processivity is a measure of the ability of RT to bind to a primer/template (P/T) and to continue to polymerize DNA without falling off the P/T. The thermodynamic drive for substrate binding in RT-catalyzed DNA synthesis is ordered and consistent with that observed with other DNA polymerases (Majumdar 1988). First, the primer and template (P/T) bind to RT. Then, dNTP binds to the P/T-RT complex forming a catalytically inactive 3' complex. This is followed by isomerization to generate a catalytically active 3' complex. This step is thought to provide the energy required for polymerization (Huber 1989, Majumdar 1988). What happens next depends on whether polymerization is processive or distributive. In

the former, HIV-1 RT travels along the P/T without interruption. In distributive polymerization, HIV-1 RT regularly detaches from the P/T and reattaches (Bryant 1983, Reardon 1990). In processive polymerization, the K_{off} (rate of P/T dissociation from the enzyme) is slow relative to the rate of translocation and the binding of the next required nucleotide. The rate determining step is the actual catalytic event and/or precatalytic conformational changes (as seen with *E coli* DNA pol I Klenow fragment (Bryant 1983, Reardon 1990). Whether reverse transcription proceeds processively or distributively depends on several factors. They include whether the reaction involves DDDP or RDDP, the nature of the P/T, whether dNTP pools are limiting, and what the kinetic parameters of the specific reaction are. An inverse relationship appears to exist *in vitro* between RT processivity and the effectiveness of nucleoside analogues. Thus, alterations in processivity are likely to affect ddNTP effectiveness. Pausing by RT could also diminish processivity. HIV-1 RT pausing has been shown to occur when RT encounters certain regions of secondary structure (that have to be melted before reverse transcription can be continued), in homopolymeric stretches of template, or during the template switches. Pausing by RT may lead to increased infidelity (Preston 1988) and, we are postulating, to greater incorporation of nucleoside analogues.

A. Preferential Chain termination of reverse transcription after the first template switch by AZT in the case of wild type HIV-1

The steady-state synthesis of (-) strong-stop DNA both *in vivo* and *in vitro* proceeds in a processive manner until reverse transcriptase reaches the 5' end of the HIV-1 LTR. RT then pauses while it switches templates from the 5' end of one (+) viral RNA strand to the 3' end of the same or the other strand of (+) viral RNA (Luo 1990, Oyama 1989, Panganiban 1988, Peliska 1992). Previous reports have documented preferential chain termination by nucleoside analogues after this first template switch in both Jurkat cells (Arts 1994c) and PHA-activated PBMC infected by wild type HIV-1 (Gelezuinas 1993). We have confirmed these observations. Figures 6 and 7 show that products are not chain terminated by AZT to a significant degree prior to the first template switch relative to the amount of chain termination that occurs after the first template switch.

This phenomenon is likely associated with pausing (decreased processivity) that is displayed by RT during the first template switch. This explains why the lack of chain termination in cell-free reactions during the synthesis of (-) strong-stop DNA and the chain termination seen thereafter when pausing resulted in incorporation of nucleoside analogues.

HIV-1 RT has also been shown to pause when synthetic primers such as deoxynucleoside DNA (dPR) are used instead of $tRNA^{Lys,3}$ (Arts 1994b). Reactions primed with dPR resulted in more chain termination probably owing to lack of stem-loop structure found in $tRNA^{Lys,3}$ that is important in template switching (Arts1994b, Isel 1993). Furthermore, synthesis of (-) HIV-1 DNA after the first template switch involves a RDDP priming event that

utilizes (-) strong-stop DNA as a primer. DNA primed DDDP is thought to be processive in nature. There is a forced termination of polymerization that results in a rate determining release of the P/T from the enzyme (Reardon 1990). DNA primed RDDP, in contrast to DNA primed DDDP, is thought to be distributive in nature (Reardon 1990). Priming of (-) strand HIV-1 DNA by (-) strong-stop DNA can be likened to a dPR priming event from the PBS which would result in increased chain termination.

These observations offer explanations for the select incorporation of nucleoside analogues and chain termination following the first template switch.

B. Nucleoside analogues are more effective chain terminators when dNTPs are limiting

HIV-1 RT also pauses when dNTP pools are limiting. RT is both less processive and slower in cells that have low dNTP pools (e.g., human fetal brain macrophages) (O'Brien 1992, Richmann 1987). In such cells, AZT is an effective inhibitor of formation of (-) strong-stop DNA (Gelezuinas 1993). In contrast, RT is more processive and faster in cells that have high dNTP pools (e.g., CD4+ Jurkat cells). In such cells, AZT does not cause chain termination prior to the first template switch (Arts 1994c).

Intracellular dNTP levels can also affect infectivity by HIV-1. dNTP levels in quiescent PBMC are lower than in PHA-activated PBMC (Balzarini 1989, Coates 1992, Dahlberg 1987). In fact, dNTP levels in quiescent cells are lower than their K_m values for RT. In contrast, they are higher than the K_ms in PHA-activated PBMC

(Gao,W-Y. 1993a). This implies that much of the catalytic potential of HIV-1 RT is wasted in quiescent cells explaining why productive infection in such cells (i.e., most of the PBMCs in the body) proceeds at a slower rate than in activated cells (Zack 1990 and 1992, Spina 1995, Gao,W-Y. 1993a). The discrepancy in dNTP pools between quiescent and PHA-activated PBMC is likely attributable to low levels of thymidine kinase and deoxycytidine kinase activities in quiescent cells relative to activated ones (Gao,W-Y.1993a).

High dNTP pools are present in activated PBMC (Gao,W-Y. 1993a), in which reverse transcription is more processive, rapid and efficient. Quiescent cells show a biphasic pattern of reverse transcription, which is initially distributive followed by an increase in and generation of full-length transcripts can be made.

We found that nucleoside analogues (AZT, ddI, and 3TC) exerted major reductions in synthesis of (-) strong stop DNA in quiescent but not PHA-stimulated PBMC, a distinction attributable to the higher dNTP pool sizes in the latter (Dahlberg 1987). Similar results have been observed with monocyte/ macrophage cultures treated with granulocyte/macrophage-colony stimulating factor (GM-CSF) (Perno 1988 and 1989). Others have shown that diminution of dNTP concentrations, enacted via treatment with hydroxyurea (HU), leads to inhibition of reverse transcription as well as to an increase in the anti-viral activity of nucleoside analogues (Dahlberg 1987, Fischl 1987, Fitzgibbon 1987, Larder 1989a). HU is an inhibitor of ribonucleotide reductase, an enzyme catalyzes the reduction of ribonucleotide to deoxyribonucleotides. These results imply that there is a strong relationship between dNTP concentration, pausing,

the extent of reverse transcription, and the inhibitory effects of nucleoside analogues.

The increased inhibition of (-) strong stop DNA seen in quiescent PBMC is likely to be due to decreased dNTP levels rather than to changes in levels of ddNTPs since the addition of nucleoside analogues to PBMC does not lead to decreased phosphorylation of native nucleoside nor to increased ddNTP concentrations (Balzarini 1989, Coates 1992, Dahlberg 1987). Furthermore, with the exception of AZT-TP, ddNTP concentrations were only slightly higher in PHA-stimulated than in quiescent PBMC, attesting to differences in phosphorylation kinetics between native nucleoside and their analogues (Balzarini 1989, Fischl 1987, Johnson 1989). The conclusion is that low dNTP concentrations alone are sufficient to explain the impairment of DNA elongation that is observed in quiescent cells (Gao,W-Y. 1993a).

We have observed chain termination in cell-free RT assays in the presence of each of ddCTP, ddATP, AZT-TP, 3TC-TP, and ddITP at 1/2 and 1/10 of native dNTP concentrations. In the case of 3TC-TP, the data obtained in cell-free assays are in contradistinction to results showing high levels of inhibition of (-) strong-stop DNA in quiescent PBMC (Gao,W-Y. 1993a and 1993b). Increased anti-RT activity of 3TC in quiescent PBMC may be due to several factors including uptake of ddN by cells (i.e. passive diffusion or active transport), stability of the compound in the cell, relative rate of phosphorylation to ddNTP, degradation of ddNTP, and the relative binding efficiency of ddNTP to the RT enzyme. In general, therefore, effective anti-viral concentrations of unphosphorylated nucleoside

analogues in tissue culture may not always correspond to effective anti-RT activities of corresponding ddNTPs in cell-free RT reactions.

The catalytic efficiency, (K_{cat}/K_m) and the maximal rate (V_{max}) of incorporation of a dNMP are significantly greater than those for a ddNMP (analogue) (Gelezuinas 1993, Mitsuya 1985). At sub-maximal rates of polymerization (i.e. pausing or at limiting dNTP concentrations), V_{max} values for incorporation of ddNMP or dNMP no longer apply. Under these conditions, incorporation of dNMP or ddNMP is likely determined by other parameters, e.g.,. binding affinities of dNTP and ddNTP to RT and efficiencies of phosphorylysis of bound dNTP or ddNTP. It has been shown that decreased dNTP concentrations may result in increased pausing by HIV-1 RT. Since pause sites are also sites of primer/template dissociation, it is not surprising that increased HIV-1 DNA synthesis occurred in PHA-stimulated PBMC compared with quiescent PBMC grown in high concentrations of dNTPs. If chain termination by nucleoside analogues is directly related to pausing by HIV-1 RT, it follows that increased pausing might augment such events. HU has been shown to decrease intracellular concentrations of dNTPs and to act synergistically with nucleoside analogues to promote inhibition of HIV-1 replication (Fitzgibbon 1992, Larder 1989a) Drugs that disrupt primer/template/HIV-1 RT complexes may also increase pausing by RT during polymerization and show synergy with nucleoside analogues. Nevirapine, synergistic with AZT in inhibition of HIV-1 replication, is thought to inhibit the binding of RT to the primer/template (Kohlstaedt 1992, Smerdon 1994).

C. The stimulatory effect of AZT on the generation of (-) strong-stop DNA following infection by AZT-resistant isolates of HIV-1

RT processivity is strongly related to the manner in which the enzyme grips the P/T during reverse transcription. The flexibility of this grip permits RT to detach from and reattach to the P/T, events important in template switching and, probably, genetic recombination as well.

Gripping the primer/template is essential for RT activity and involves the positioning by RT of the free 3'-hydroxyl of the primer over the *pol* active site of the palm subdomain (Jacob-Molina 1993). Amino acids 195-300 of the palm and thumb subdomains are involved in this primer binding (Kohlstaedt 1992). Other regions of RT are thought to contact nucleic acid and to contribute to the P/T grip. Amino acids 227-235, for example, represent a part of the enzyme that grips the primer sugar-phosphate backbone (Davies 1991, Jacob-Molina 1993).

We observed that AZT treatment of both Jurkat cells (data not shown) and of PHA-activated PBMC (Figure 11 and 12) prior to infection with AZT-resistant clinical isolates of HIV-1 resulted in increased levels of (-) strong stop DNA relative to levels in cells not treated with drug and in cells infected by wild-type virus. Furthermore, figure 13 shows that at low AZT concentrations ddI-sensitive/AZT-resistant viruses were rendered resistant to ddI.

Interestingly, most mutations that confer AZT-resistance (41, 67, 215, 219) are located in the "finger" and "thumb" sub-domains of

RT essential for gripping the P/T. All the AZT-resistant viruses used here had at least one major mutation in this region (D'Aquila 1994). It is possible that RT processivity may be greater for enzymes that are mutated at these site, perhaps due to enhanced clamping of the template to the enzyme. We found that the stimulatory effect on generation of (-) strong-stop DNA was observed only with AZT-resistant isolates in the presence of AZT. Conceivably, AZT may enhance the template stability in the P/T grip of AZT-resistant isolates leading to more reverse transcription and generation of DNA. This could account for the drug-resistance phenotype.

AZT may also alter the conformation of mutated RT leading to enhanced synthesis of (-) strong-stop DNA, an effect not seen in the absence of the drug. Free DNA found in the "B"-form resembles a stretched "A"-form, but has a wider major groove, 11 bases per turn and a pitch of approximately 34 angstrom (Dickerson 1982). Interaction with wild type RT induces an alteration of the P/T to an "A"-like-form, allowing for an optimal phosphate groove separation between bases for nucleophilic attack by the RNaseH active site on the RNA strand (Federoff 1993, Yu 1993). In the connection subdomain (about 10 nt into the ds DNA) an "A" -> "B" form switch occurs in the ds DNA (Jacob-Molina 1993, Metzer 1993). Contacts between HIV-1 RT and nucleic acid P/T may induce torsional and conformational changes, that enable reverse transcription to occur. The fingers mutations that confer AZT-resistance are thought to alter the allowable bend (45' angle) between "A" form nucleic acid in the polymerase domain and 'H' form nucleic acid in the RNaseH domain of RT (Japour 1995). This alteration may allow RT to alter its grip on

of RT. This alteration may allow RT to alter its grip on the P/T, such that there is increased generation of (-) strong stop DNA (D'Aquila).

This might also explain why high levels of RT activity in culture fluids of CD4+ cells infected with AZT-resistant (ddI-sensitive) HIV-1 in the presence of otherwise inhibitory concentrations of ddI. It is possible that low concentrations of AZT may enhance reverse transcription to a greater degree than ddI inhibits it. Alternatively, it is also possible that there may be a competition that is occurring between AZT and ddI in the resistant isolates of HIV-1 for binding to the RT or in the perturbations of the cells' dNTP pools. In either case, more work is required to shed light on the observation that AZT-resistant HIV-1 seems to be able to replicate in the presence of ddI when there is a small concentration of AZT present, as opposed to when AZT is absent.

This effect is not seen with 3TC-resistant viral clones (figures 14 and 15) in the presence of either AZT or 3TC and, thus, may be unique to AZT-resistant viruses. As stated, different mechanisms may account for resistance to individual nucleosides or classes of drugs. For example, ddI-resistance appears to be associated with an alteration of the orientation of the primer's terminus such that there is greater differentiation between ddI and the natural substrate (D'Aquila) while AZT-resistance appears to be associated with mutations in the fingers' domain of RT. Conclusions drawn about resistance to one drug cannot necessarily be made about resistance to another drug. Once again, this can be attributable to the fact that all of these drugs have different effects on HIV-1 RT, cellular dNTP pools and phosphorylation pathways, and they have different kinetic

constants. The fact that there are different mutations that arise for the different drugs reinforces the fact that what may be true for one drug does not necessarily hold true for another. Furthermore, the fact that different mutations arise in response to different drugs supports the idea that these drugs interact with HIV-1 and HIV-1 RT in different manners. Interestingly, combinations of AZT with FLT or ddI were synergistic in inhibition of AZT-sensitive viruses but additive and/or antagonistic in regard to AZT-resistant viruses (Cox 1992b) and (Cox 1993). Combinations of atervidine, a non-nucleoside reverse transcriptase inhibitor (NNRTI) to which AZT-resistant and ddI-resistant HIV-1 are both sensitive, and AZT were synergistic in inhibition of AZT-resistant HIV-1 but only additive in regard to AZT-sensitive virus. We believe that mutations that confer AZT-resistance cause conformational changes in RT resulting in increased processivity when AZT is present and that this may constitute an important mechanistic aspect of AZT drug-resistance.

D. Conclusion

Further work is now needed to define the events in reverse transcription and to compare drug-resistant and drug-sensitive recombinant molecules. Although resistant viruses may be as infectious as parental isolates (Wainberg 1992), it is also true that resistant viruses lack any growth advantage in the absence of drug pressure (Wainberg drugs under experimental and clinical research 1992). Studies on AZT-resistant and -sensitive recombinant RTs failed to demonstrate differences in either kinetic (K_m, V_{max}) or

inhibition (K_i) constants or in catalytic efficiency (V_{max}/K_m) (Dianzani 1992, Eisenthal 1974, Larder 1989a, Pokhlok 1993, Wainberg 1990). However, AZT-resistant RTs are more processive than their wild type counterparts (D'Aquila - sardinia conference). Further work on drug-resistance and new drug development will hopefully lead to more effective control of HIV-1 replication and pathogenesis.

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