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Convergent Evolution of Reverse Transcriptase (RT) Genes of Human Immunodeficiency Virus Type 1 Subtypes E and B following Nucleoside Analogue RT Inhibitor Therapies

HIRONORI SATO,[1]* YASUHIRO TOMITA,[1] KAYO SHIBAMURA,[1] TEIICHIRO SHIINO,[1] TUYOSHI MIYAKUNI,[2] AND YUTAKA TAKEBE[1]

Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Shinjuku, Tokyo 162-8640,[1] and Naha Prefectural Hospital, Naha, Okinawa 902,[2] Japan

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Changes in the drug susceptibility, gene lineage, and deduced amino acid sequences of the reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) subtype E following 3′-azido-3′-deoxythymidine (AZT) monotherapy or AZT–2′,3′-dideoxyinosine combination therapy were examined with sequential virus isolates from a single family. The changes were compared to those reported for HIV-1 subtype B, revealing striking similarities in selected phenotype and amino acids independent of differences in the RT backbone sequences that constantly distinguish the two subtypes. Particularly, identical amino acid substitutions were present simultaneously at four different positions (D67N, K70R, T215F, and K219Q) for high-level AZT resistance. These data suggest that HIV-1 subtypes E and B evolve convergently at the phenotypic and amino acid levels when the nucleoside analogue RT inhibitors act as selective forces.

Human immunodeficiency virus type 1 (HIV-1) has the error-prone RNA-dependent DNA polymerase, thereby circulating in vivo as a quasispecies, which in turn rapidly generates the variants resistant to anti-HIV drugs. Mutations associated with drug resistance were first identified in the reverse transcriptase (RT) genes of virus isolates selected by 3′-azido-3′-deoxythymidine (AZT) therapy. The AZT-resistant isolates possess combinations of four amino acid substitutions encoded by an RT gene (D67N, K70R, T215F, and K219Q), which confer, if present simultaneously, high levels of AZT resistance on the sensitive clone (23, 24). Subsequently, two mutations (M41L and L210W) in other strains have been shown to contribute to AZT resistance (13, 14, 17), suggesting the presence of variations in AZT-resistant mutations among HIV-1 strains.

HIV-1 strains circulating in different geographic locations are classified into multiple genetic subtypes (A to H, N, and O) on the basis of sequence variations of the *gag* and *env* genes (19). Each subtype represents a distinct HIV-1 quasispecies (26) and possesses unique amino acids in the RT backbone sequence (19). HIV-1 subtype E is a regional variant that has been found to cause the AIDS epidemic in Southeast Asia (16, 20, 21, 34). The RT sequence of subtype E differs from that of subtype B in Europe and North America by about 10% of its nucleotides and 7% of its amino acids (19). The differences may cause changes in the patterns of amino acid substitution or in local conformations of the RT protein, which in turn may result in subtype-dependent variations in resistance mutations for RT inhibitors. However, these issues remain unclear because phenotype and sequence changes during antiviral therapy have been reported exclusively with subtype B.

We recently reported a family case in which a single subtype E virus source diversified into variants with distinct biological phenotypes, providing a model for subtype E evolution in vivo (29, 30, 32). In this study, we examined changes in drug susceptibility and RT gene sequences of the virus isolates from the family following AZT monotherapy or AZT–2′,3′-dideoxyinosine (ddI) combination therapy. The data, which provided the first RT sequence of a subtype E AZT-resistant variant with information regarding the genotypic and phenotypic changes after RT inhibitor therapy, were used to assess whether HIV-1 subtype E and B evolve convergently or divergently under the selective pressures of nucleoside analogue RT inhibitors. The findings obtained there have implications for studies of evolution, molecular mechanisms, and evaluation systems of HIV-1 drug resistance.

Clinical information of the family. The family consisted of a male index patient (NH1), the female spouse of NH1 (NH2), and their child (NH3). Although NH1 was treated with AZT (400 mg per day) in October 1992, the therapy was discontinued in November 1992 due to poor compliance. NH1 developed AIDS (Centers for Disease Control and Prevention [CDC] category C3) at the time of blood collection in June 1993 and died of AIDS-related pulmonary complications in March 1994. NH2 was asymptomatic (CDC category A2) at the initial blood collection in June 1993. NH2 developed AIDS-related *Pneumocystis carinii* pneumonia (CDC category C3) in February 1996. NH2 was treated with AZT (400 mg per day) in April 1996 and then with AZT (300 mg per day) plus ddI (200 mg per day) in May 1996. Follow-up blood collection was done for NH2 in March 1996 (NH2-II) and January 1997 (NH2-III), 1 month before and 9 months after the therapy, respectively. NH2 died of AIDS-related neurologic complications affecting the brain in December 1998. NH3 had no AIDS-defining illness and no history of anti-HIV treatment until the time of blood collection in June 1993.

Drug susceptibility of HIV-1 subtype E virus isolates from the family. Five HIV-1 subtype E strains, HIV-1_{NH1}, HIV-1_{NH2}, HIV-$1_{NH2\text{-}II}$, HIV-$1_{NH2\text{-}III}$, and HIV-1_{NH3}, were isolated at each sampling point described above (29). The sensitivity of each virus isolate to AZT or ddI was examined in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PHA-PBMCs) (5). Because HIV-1_{NH2} and HIV-1_{NH3} repli-

* Corresponding author. Mailing address: Laboratory of Molecular Virology and Epidemiology, AIDS Research Center, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku, Tokyo 162-8640, Japan. Phone: (81)-3-52851111. Fax: (81)-3-52851129. E-mail: hirosato@nih.go.jp.

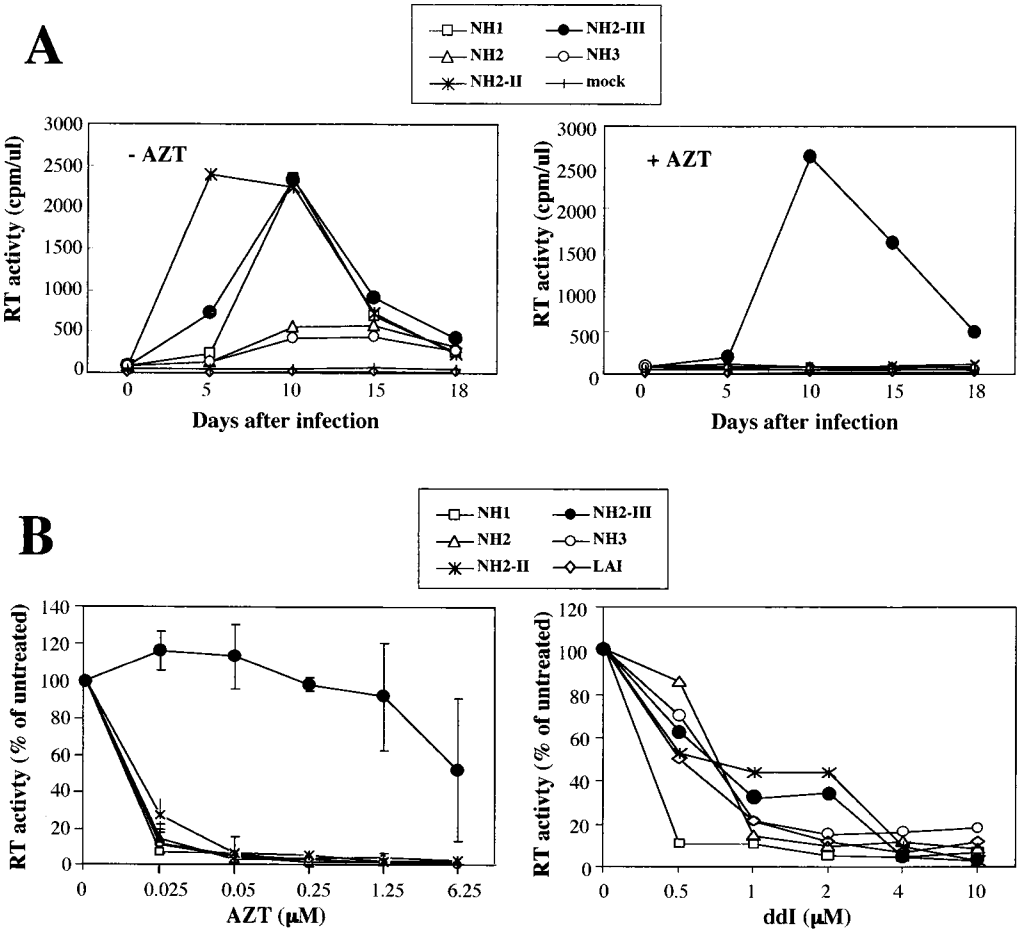

FIG. 1. AZT and ddI susceptibility of HIV-1 subtype E virus isolates from a family. (A) Replication kinetics. PHA-PBMCs were infected with virus stock (100 $TCID_{50}$) and cultured in the absence (left panel) or the presence (right panel) of 6.25 μM AZT. RT activity (35) in the culture medium was monitored at the indicated time points. (B) Dose-response curve. Infected cells were cultured in the indicated concentrations of AZT (left panel) or ddI (right panel). RT activity at the peak of infection is expressed as a percentage of that of the untreated culture. Mean values with the standard errors of the three independent experiments for AZT and mean values of quadruplicates of an experiment for ddI are plotted.

cated poorly in the PHA-PBMCs, PBMCs were depleted of $CD8^+$ cells with anti-CD8 antibody and a magnetic cell sorter (Miltenyi Biotec, Bergisch-Gladbach, Germany), and the CD8-positive-cell-depleted PHA-PBMCs were used for the present assay. The 50% tissue culture infective dose ($TCID_{50}$) of each virus stock was determined by the endpoint dilution assay. The PHA-PBMCs were incubated in virus stock (100 $TCID_{50}$) for 3 h, washed twice, and cultured (4×10^5 cells in quadruplicate in a 96-well plate) for 18 days in various concentrations of AZT (0, 0.025, 0.05, 0.25, 1.25, and 6.25 μM) or ddI (0, 0.5, 1.0, 2.0, 4.0, and 10.0 μM). The RT activity (35) of the culture supernatant was monitored every 3 to 5 days during the 18-day infection periods.

Figure 1A shows the replication kinetics of the NH viruses in the absence or presence of 6.25 μM AZT. Without AZT, HIV-1_{NH1}, HIV-1_{NH2-II}, and HIV-$1_{NH2-III}$ replicated with faster kinetics to much higher titers than HIV-1_{NH2} and HIV-1_{NH3} did (Fig. 1A, left panel). The AZT (6.25 μM) effectively blocked progeny virus production in most of the NH virus isolates except for HIV-$1_{NH2-III}$ (Fig. 1A, right panel). The HIV-$1_{NH2-III}$ started to produce detectable RT activity with slightly slower kinetics than it did without AZT, peaking on day 10 after infection.

AZT concentrations inhibiting RT activity to 50% of the peak activity of the drug-free control (IC_{50}) were determined on the basis of the dose-response curve shown in the left panel of Fig. 1B. HIV-1_{NH1}, HIV-1_{NH2}, HIV-1_{NH2-II}, and HIV-1_{NH3} were as sensitive to AZT as the subtype B control strain (HIV-1_{LAI}); IC_{50} for these strains were less than 0.025 μM. In contrast, HIV-$1_{NH2-III}$ exhibited high-level resistance to AZT, with the IC_{50} being approximately 6 μM (>240-fold increase) (Fig. 1B, left panel), a value similar to those reported for highly resistant subtype B virus isolates (1, 12, 23–25). The IC_{50} values of ddI were similar for the NH viruses and HIV-1_{LAI}, ranging from 0.35 to 0.8 μM (Fig. 1B, right panel). These data were consistent with the observation that the different subtypes of major group (group M) HIV-1 (19) exhibited similar susceptibilities to AZT or ddI when the viruses were from individuals receiving no antiviral drugs (27). Furthermore, the phenotypic change in the NH2 viruses was very similar to that seen in HIV-1 strains circulating in Europe and North America, supporting the notion (4, 25, 31) that AZT-ddI combination therapy often selects AZT-resistant variants lacking ddI resistance.

Molecular evolution of the RT gene sequences of the subtype E virus isolates. Viral RNA was extracted from each virus

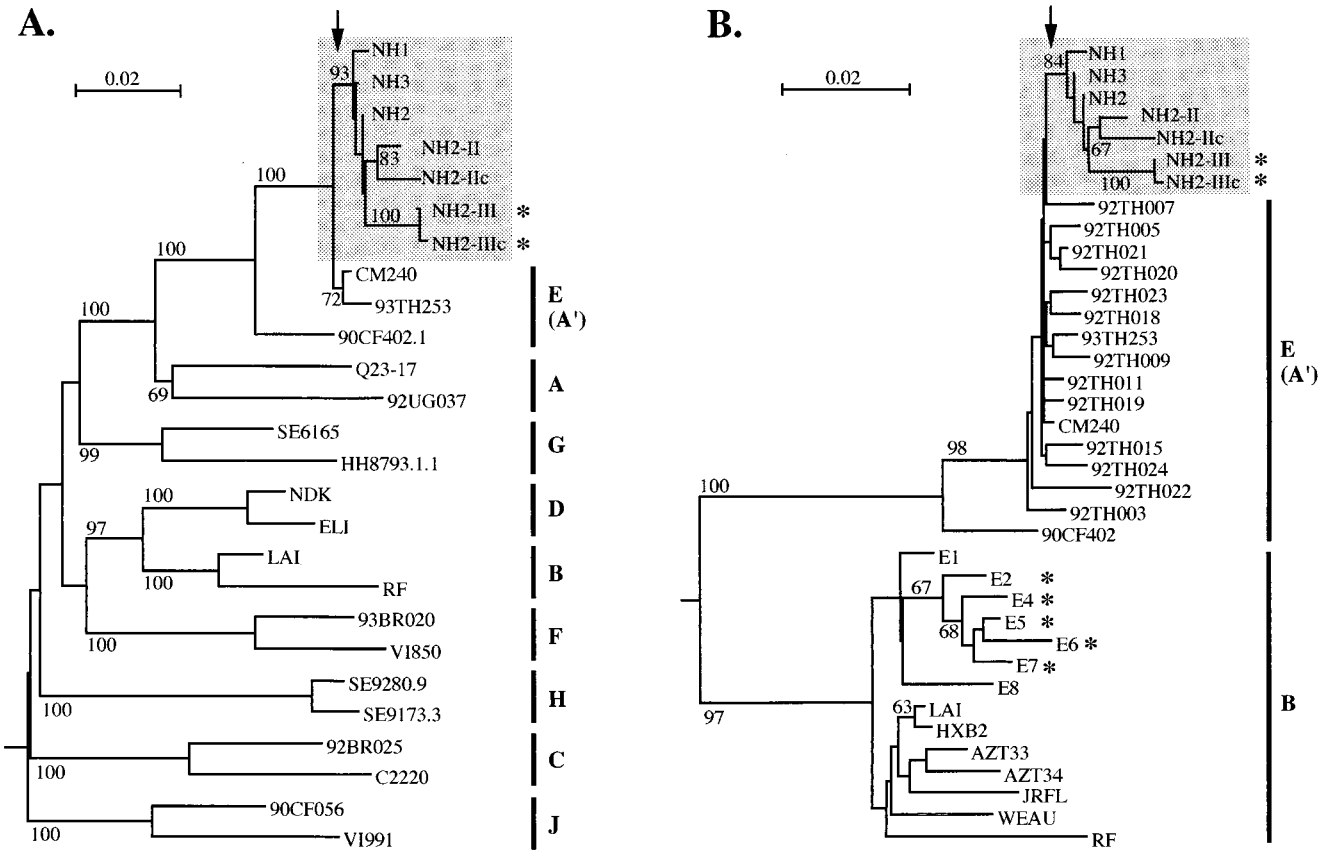

FIG. 2. Neighbor-joining trees of the nucleotide sequences of the RT gene segments (738 bp). The trees were constructed with CLUSTAL W and programs of the PHYLIP package and rooted with the homologous region of a chimpanzee's simian immunodeficiency virus variant ($SIV_{CPZ}GAB$). (A) Comparison of the family virus samples with HIV-1 group M references (subtypes A to J) (19). (B) A comparison with subtype E (8, 10, 11) and subtype B (E1 to E8) (12) samples with differences in AZT sensitivity. Bootstrap values above 60/100 are indicated at the nodes of the trees. Symbols: shaded box, family virus samples (NH1, NH2, NH2-II, NH2-III, and NH3 [direct sequence samples]; NH2-IIc and NH2-IIIc [clonal sequences]); arrows, the branches that lead to the family virus cluster; *, sequences from virus isolates with an AZT resistance phenotype.

stock with a High Pure viral RNA kit (Boehringer GmbH, Mannheim, Germany). A DNA segment (1,011 bp) encoding the carboxyl-terminal end of HIV protease and the amino-terminal half of HIV RT was amplified by RT-PCR with a TaKaRa One Step RNA PCR kit (Takara Shuzo, Otsu, Japan). The primer pair used in the amplification was ERT327A (5′-GTG GAA AAA AGG CTA TAG GTA CAG-3′) and ERT328B (5′-CTG CCA ACT CTA ATT CTG CTT C-3′). The PCR products were purified with Centricon-100 (Amicon, Danvers, Mass.) and used for direct sequencing on an ABI PRISM310 automated DNA sequencer (Perkin-Elmer, Foster City, Calif.). The primers used in the sequence reaction were ERT327A, ERT328B, ERT329A (5′-ACT CAG GAC TTT TGG GAA GTT C-3′), and ERT330B (5′-GAT CCT ACA TAC AAG TCA TCC-3′). For the HIV-1_{NH2-II} and HIV-$1_{NH2-III}$ viruses, a portion of the PCR products was cloned into the pCRII cloning vector (Invitrogen, Carlsbad, Calif.), and clonal sequences were also obtained. All the NH virus nucleotide sequences contained an open reading frame encoding the conserved YMDD polymerase motif. The nucleotide sequences differed from those of subtype E strains from Thailand (8, 10, 11), a subtype E strain from Africa (11), and subtype B strains (19) by approximately 1.7, 4.6, and 9.0 to 11.8, respectively.

The nucleotide sequences encoding the amino-terminal half of RT (738 bp) were aligned with HIV-1 subtype references by using CLUSTAL W version 1.74 (33). The neighbor-joining trees with bootstrap values of 100 resamplings were constructed with NEIGHBOR, DNABOOT, and CONSENSE programs of the PHYLIP package, version 3.5c. Figure 2A shows the genetic relationship of the NH virus RT sequences with HIV-1 group M references (subtypes A to J) (19). The overall topology of the tree was very similar to that obtained with the NH virus *gag* sequences (29); the NH samples formed a monophyletic group (bootstrap value, 93/100) that was most closely related to the subtype E samples from Thailand (93TH253 and CM240) and then to the subtype E sample from the Central African Republic (90CF402.1). The subtype E samples formed a single cluster (bootstrap value, 100/100) related to subtype A sequences as reported previously (10). Consistent with the family's epidemiologic data (29), NH virus samples from 1993 (NH1, NH2, and NH3) were very closely related to a putative ancestor of the intrafamilial infection at the node of the family cluster (Fig. 2). These data suggest that the RT genes of the NH viruses evolved from a common subtype E ancestor RT sequence of Thai origin that was distant from the subtype B RT gene lineage, supporting our previous notion regarding the origin of the NH viruses (29).

Figure 2B shows the genetic relationship of the RT gene sequences of subtype E and B virus isolates with the difference in AZT sensitivity. The monophyletic relationship and the branching pattern of the NH samples were reproducible when

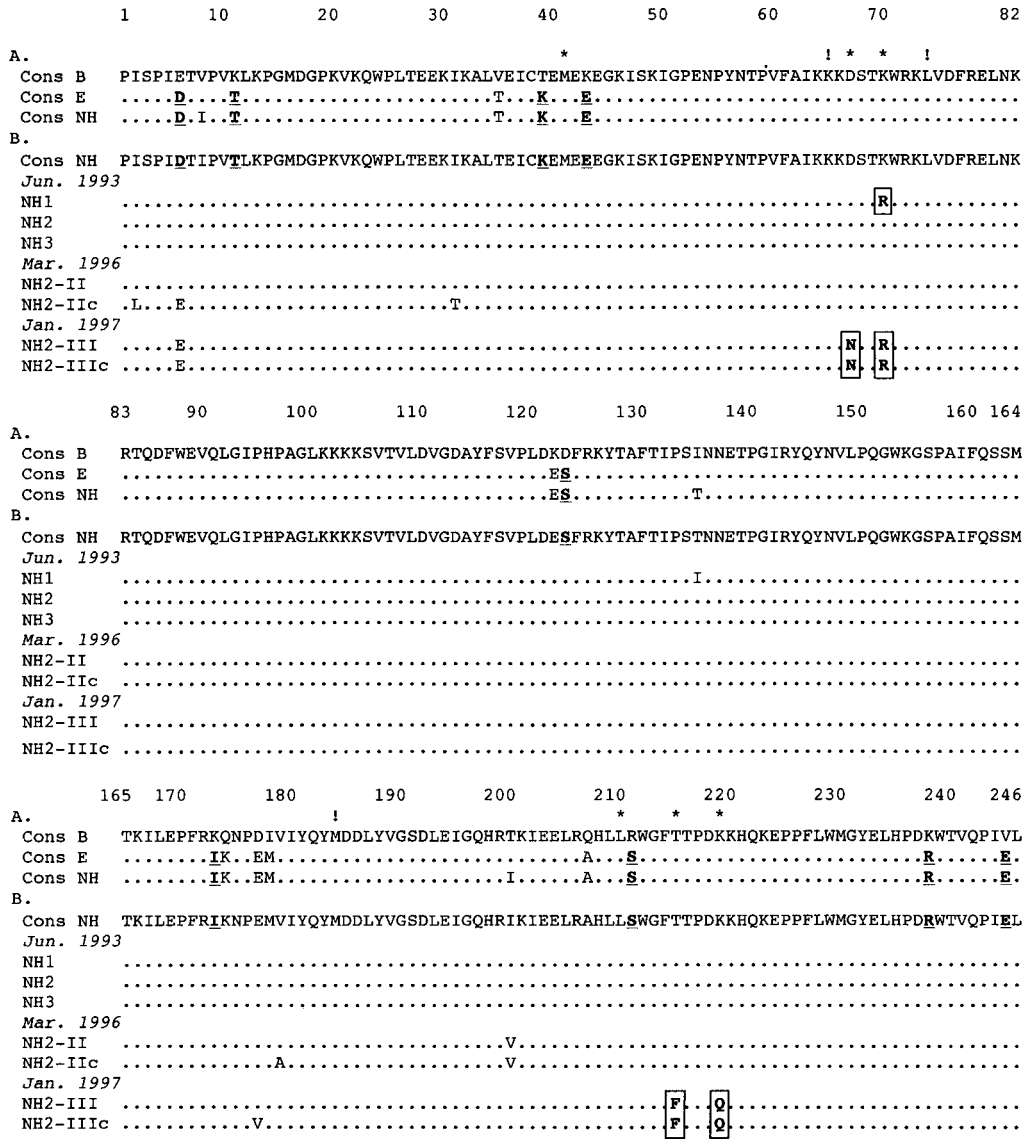

FIG. 3. Alignment of the deduced amino acid sequences of the amino-terminal half of the HIV-1 RT p66 protein (positions 1 to 246). (A) A family virus consensus sequence of the 7 virus sequences (Cons NH) is aligned with a consensus of the 16 available subtype E sequences (Cons E) (8, 10, 11) and a consensus of 32 subtype B sequences (Cons B) (19). Symbols: dashes, identity with Cons B; * and !, positions in the gene associated with AZT and ddI resistance in subtype B strains, respectively; underlined bold letters, amino acids found exclusively in subtype E or in subtype E and A strains. (B) Each of the virus sequences is aligned under Cons NH. Symbols: dashes, identity with the Cons NH; open boxes, amino acids associated with an AZT resistance phenotype in subtype E and B strains.

more subtype E and B samples with different AZT sensitivities were included in the tree. Thus, as expected, the selection pressure of AZT did not change subtype identity of RT gene; rather, it selected RT sequences that were highly related to their drug-sensitive predecessors.

Characteristics of primary and secondary structures of RT proteins of the subtype E virus isolates. The characteristics of the deduced amino acid sequence of the amino-terminal half of the NH virus RT protein (amino acids 1 to 246) were examined. The NH virus consensus differed from the subtype E and B consensuses at 3 (1.2%) and 18 (7.3%) of the 246 residues, respectively (Fig. 3A). Among the 18 amino acid substitutions between the NH virus and subtype B consensus, 15 were seen in a comparison between subtype E and B consensuses (Fig. 3A). Out of these, seven amino acids (D6, T11, K39, E43, I173, R238, E245) and two amino acids (D123S, S211) have been reported to occur exclusively in subtype E and in subtypes E and A (19), respectively, constituting a possible amino acid signature specific to the subtype E RT gene.

The 15 amino acid differences between subtype E and B consensuses indicate potential residues that may cause differences in the secondary structure of RT proteins of the two subtypes. First, nine substitutions (K11T, T39K, K43E, K122E, D123S, K173I, Q174K, R211S, and V245E) were predicted to introduce changes in the positive or negative net charge around the positions. Second, in a comparison of the predicted secondary structures (9), the K122E substitution caused a loss of a β-turn structure in the subtype B consensus, while R211S generated an alternative β-turn in the NH virus or subtype E consensus. Third, in a comparison of the predicted hydrophobicity profile (22) of the consensus sequences, six substitutions (K11T, V35T, K1731, T200I, Q207A, and V245E) caused sig-

nificant changes in the hydrophobic character of RT subdomains in the NH or subtype E consensus.

None of the 18 differences between the NH virus and subtype B consensus (Fig. 3A) or of the differences between subtype E and B strains (8, 10, 11) have been reported to confer drug resistance to current RT inhibitors in subtype B strains. These data and the predominance of AZT-sensitive strains in subtype E (Fig. 1) (27) suggest that the subtype E RT protein generally has a structure sensitive to the RT inhibitors. On the other hand, the above-mentioned data indicate that subtype E RT inherits amino acids that are distinct from those of subtype B RT and may cause local modifications in p66 subdomains.

Amino acid substitutions associated with AZT therapy. Amino acid substitutions associated with AZT resistance were examined by comparing seven NH virus RT sequences (Fig. 3B). The direct sequence sample of HIV-$1_{NH2\text{-}III}$ showed five substitutions (D6E, D67N, K70R, T215F, and K219Q) compared with the NH virus consensus, the AZT-sensitive ancestor of HIV-$1_{NH2\text{-}III}$, or the sibling. The clonal sequence of the HIV-$1_{NH2\text{-}III}$ also had these five mutations, confirming that the substitutions were present simultaneously in a single RT gene of HIV-$1_{NH2\text{-}III}$. The D6E, D67N, K70R, and K219Q substitutions involved a single nucleotide substitution (GAC to GAA, GAC to AAC, AAA to AGA, and AAA to CAA, respectively), while the T215F substitution required two base changes (ACT to TTT).

Three of the five substitutions (D67N, T215F, and K219Q) in HIV-$1_{NH2\text{-}III}$ were never seen in the AZT-sensitive NH virus isolates (Fig. 3B) or in the 16 subtype E isolates from individuals in early 1990 (8, 10, 11). The K70R substitution was seen also in the virus isolates from the individual who had a history of receiving AZT (HIV-1_{NH1}). These data suggest that the four substitutions (D67N, K70R, T215F, and K219Q) have a strong association with AZT therapy. In contrast, the D6E substitution was seen in a clonal sequence (NH2-IIc) of an AZT-sensitive NH virus and in many of the AZT-sensitive subtype B isolates (Fig. 3B) (19), suggesting that this substitution can occur independently of drug treatment. The RT genes of the NH viruses had no substitutions for the resistance to ddI nor for the multiple drug resistance to AZT and ddI (Fig. 3B), which is consistent with the lack of ddI resistance of the NH viruses (Fig. 1B).

Potential roles of the amino acid substitutions in AZT resistance and RT gene evolution of subtype E. The changes in drug susceptibility and amino acid sequence of RT in the NH family were compared to those reported for HIV-1 subtype B. Notably, the positions and types of four of the five amino acid substitutions in the HIV-$1_{NH2\text{-}III}$ were completely identical to those (D67N, K70R, T215F, and K219Q) reported for the subtype B variants exhibiting high-level resistance to AZT (23, 24) (Fig. 3B). This finding and the phenotypic similarity of the virus to subtype B with these mutations (Fig. 1) reemphasize the crucial roles of the four amino acid substitutions for HIV-1 AZT resistance and suggest that the amino acids at these positions play analogous roles in the DNA synthesis of subtypes E and B. In subtype B RT, these amino acids are clustered closely together in the p66 subunit of RT near the incoming nucleoside triphosphate (15) and play key roles in determining the processivity of DNA polymerase (2, 6, 7). It is conceivable that the three-dimensional positions and the roles of these amino acids in DNA synthesis are conserved in subtype E as well. Because the amino acid types are highly conserved at these positions in subtypes E and B (Fig. 3) (19), these loci are likely to be subjected to purifying selection in the environment without the RT inhibitors. In the presence of AZT, however, the four mutations can probably increase the fitness of viruses via an increased processivity of viral DNA synthesis, thereby remaining present in the drug-treated patient by positive selection.

The K70R substitution occurs in the finger subdomain of RT protein (18) shortly after the start of AZT therapy in subtype B infection (3). The K70R substitution was seen consistently in HIV-1_{NH1}, from the individual who received AZT for only 1 month (Fig. 3B). However, the AZT resistance of HIV-1_{NH1} was not detected in the present study (Fig. 1). In this regard, high-level AZT resistance requires combinations of multiple resistance mutations, such as T215F and K219Q mutations in the palm subdomain of p66 RT (23, 24). Consistently, the T215F substitution in HIV-$1_{NH2\text{-}III}$ required two base changes (ACT to TTT), suggesting a positive role for the phenylalanine present at position 215 in AZT resistance of subtype E. On the other hand, accumulation of secondary mutations requires several months or more of therapy (3, 14), while the NH1 patient had received AZT no longer than 1 month. Therefore, the AZT sensitivity of HIV-1_{NH1} is likely to be a result of a lack of secondary mutations that are located in the palm subdomain and can cooperate with the 70R mutation.

Clinical implications. In Southeast Asia, where subtype E infections are spreading rapidly, AZT and ddI monotherapy have been alternatives to the highly active antiretroviral combination therapy. Therefore, it is crucial to collect data on AZT and ddI resistance mutations in subtype E strains to assess drug efficacy in these areas, since currently available information on drug resistance mutations is based only on subtype B mutants. The present study reports the first RT sequence of the AZT-resistant subtype E variant, with information regarding the genetic and phenotypic changes occurring after RT inhibitor therapy. These data should provide a basis for developing systems for the evaluation of AZT resistance in subtype E strains. In addition, the NH viruses that have been characterized biologically and genetically (28–30, 32) would be useful as subtype E reference strains in the study of HIV-1 drug resistance in tissue culture.

Evolution of HIV-1 subtypes E and B. In this study, we have shown that the subtype E RT is genetically distant from subtype B RT, independent of AZT therapy, and has a conserved amino acid signature that may cause modifications in the local conformation of the RT p66 subdomain. Despite the differences in the RT backbone sequences, the changes in drug susceptibility and amino acids following AZT monotherapy and AZT-ddI combination therapy have been shown to be strikingly similar in the two subtypes. Particularly, identical amino acid replacements were seen concurrently at four different positions (D67N, K70R, T215F, and K219Q) for high-level AZT resistance. These findings support a model in which subtypes E and B evolve convergently at the phenotypic and amino acid levels when AZT or AZT-ddI acts as a selective force. This in turn suggests the presence of common molecular mechanisms for subtypes E and B to counter selective forces by the nucleoside analogue RT inhibitors. A convergent evolution between subtypes E and B is also seen in the hypervariable V3 loop element of HIV-1 *env* gp120. Despite an approximately 40% difference in the V3 amino acids between the two subtypes, basic amino acid substitutions occur at identical positions in the loop during disease progression in association with a shift of viral coreceptor usage from CCR5 to CXCR4 (28, 29, 32). Thus, HIV-1 subtypes E and B appear to often adopt the same amino acid substitutions in order to adapt to in vivo environmental changes, even if their genes evolve divergently along each subtype lineage.

Nucleotide sequence accession numbers. The nucleotide sequences of the subtype E RT genes reported in this study have

been deposited in the DDBJ database under accession no. AB038655 through AB038661.

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