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In Vitro Enzymatic Activity of Human Immunodeficiency Virus Type 1 Reverse Transcriptase Mutants in the Highly Conserved YMDD Amino Acid Motif Correlates with the Infectious Potential of the Proviral Genome

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Reverse transcriptases contain a highly conserved YXDD amino acid motif believed to be important in enzyme function. The second amino acid is not strictly conserved, with a methionine, valine or alanine occupying the second position in reverse transcriptases from various retroviruses and retroelements. Recently, a 3.5-Å (0.35-nm) resolution electron density map of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase positioned the YMDD motif within an antiparallel β-hairpin structure which forms a portion of its catalytic site. To further explore the role of methionine of the conserved YMDD motif in HIV-1 reverse transcriptase function, we have substituted methionine with a valine, alanine, serine, glycine, or proline, reflecting in some cases sequence motifs of other related reverse transcriptases. Wild-type and mutant enzymes were expressed in *Escherichia coli*, partially purified by phosphocellulose chromatography, and assayed for the capacity to polymerize TTP by using a homopolymeric template [poly(rA)] with either a DNA [oligo(dT)] or an RNA [oligo(U)] primer. With a poly(rA) · oligo(dT) template-primer, reverse transcriptases with the methionine replaced by valine (YVDD), serine (YSDD), or alanine (YADD) were 70 to 100% as active as the wild type, while those with the glycine substitution (YGDD) were approximately 5 to 10% as active. A proline substitution (YPDD) completely inactivated the enzyme. With a poly(rA) · oligo(U) template-primer, only the activity of mutants with YVDD was similar to that of the wild type, while mutants with YADD and YSDD were approximately 5 to 10% as active as the wild-type enzyme. The reverse transcriptases with the YGDD and YPDD mutations demonstrated no activity above background. Proviruses containing the reverse transcriptase with the valine mutation (YVDD) produced viruses with infectivities similar to that of the wild type, as determined by measurement of p24 antigen in culture supernatants and visual inspection of syncytium formation. In contrast, proviruses with reverse transcriptases containing the YADD and YSDD mutations were less infectious than wild-type virus. These results point to the critical role of methionine of the YMDD motif in the activity of HIV-1 reverse transcriptase and subsequent replication potential of the virus.

An early step in the replication of the human immunodeficiency virus type 1 (HIV-1) is the reverse transcription of the single-stranded viral RNA genome into a linear, double-stranded DNA molecule (8, 55). The reverse transcription reaction is catalyzed by a virally encoded RNA-dependent DNA polymerase termed reverse transcriptase (RT) (3, 15, 53). Reverse transcription utilizes a cellular tRNA molecule hybridized at a position near the 5′ end of the RNA genome as a primer to copy the genomic RNA (51). A complex process of template switching by the RT allows the completion of the first DNA strand and the subsequent synthesis of the second cDNA strand to generate a complete copy of the viral genome sufficient for integration into the host chromosome (14, 17, 42, 52, 54, 55). Because of the nature of the reverse transcription reaction, RT must have the capability to polymerize deoxynucleoside triphosphates by using either RNA or DNA primers on RNA or DNA templates (3, 14, 46).

The critical role of RT in the replication of HIV-1 has focused considerable attention on the structural features of this protein. The amino acid sequences of numerous viral and cellular polymerases have been compared, leading to the identification of several conserved regions (2, 23, 25, 33, 43). The most highly conserved of these is a YXDD amino acid motif that is believed to be essential for polymerase function (2, 25). A similar motif, YGDTDS, is also highly conserved among many DNA-dependent DNA polymerases (57). On the basis of molecular-modeling studies of polymerases, this motif has been postulated to be at or very near the active site and possibly involved with template recognition or metal ion binding (Mg^{2+} or Mn^{2+}) required for enzyme activity (2, 5, 10, 16, 25, 39). In many RTs, including that of HIV-1, the conserved motif consists of a core sequence of four amino acids, YMDD. However, murine leukemia virus (50) and feline leukemia virus (11) have the sequence YVDD, while recently described RTs from *Escherichia coli* (30) and *Myxococcus xanthus* contain YADD (19, 29).

Previous studies have described single amino acid substitutions in the conserved YXDD region of various RTs and RNA polymerases which resulted in enzymes with drastically reduced activity, thus confirming the significance of this motif for polymerase function (18, 20, 31, 32, 34, 37). However, no studies to date have examined the significance of heterogeneity of the second amino acid of this motif with respect to RT function. To further explore this, we have utilized site-directed mutagenesis to change methionine to valine, alanine, serine, glycine, or proline. The RT gene of HIV-1 is positioned between the viral protease and endonu-

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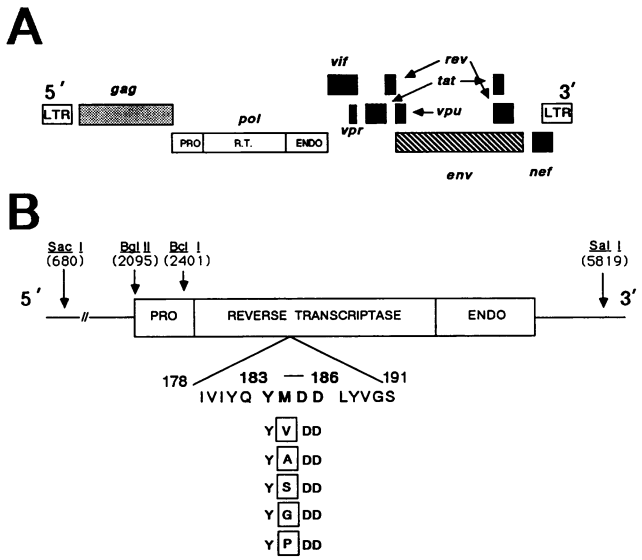

FIG. 1. Organization of HIV-1 genome and mutations in the YMDD motif of RT. The region of the *pol* gene with the RT is highlighted. (A) Protein-coding region of the HIV-1 genome, depicting *gag*, *pol*, and *env* as well as accessory genes. (B) Mutations in the YMDD region were generated by oligonucleotide site-directed mutagenesis. Substitutions changed methionine to valine, serine, alanine, glycine, or proline. Each of the mutations was subcloned into p*trp* and pHXB2 *gpt*. Numbers are nucleotides as described by Ratner et al. (44). Relevant restriction enzymes, *Bgl*II, *Bcl*I, and *Sal*I, used for subcloning of mutant RT genes into the prokaryotic expression plasmid p*trp* and an infectious HIV-1 provirus pHXB2 *gpt* are shown. PRO, protease; ENDO, endonuclease; LTR, long terminal repeat.

clease in the *pol* gene (35) (Fig. 1A). A region of the HIV-1 genome contained in a *Sac*I-*Sal*I restriction fragment (nucleotides 680 through 5819) from plasmid pBH10 (49) was subcloned into the phagemid pUC119 (59) as previously described (38). The resulting plasmid, pUC119 *Sac-Sal*, was transformed into competent *E. coli* CJ236, a *dut ung* double-mutant bacterial strain that allows uracil to be incorporated into replicated DNA at some thymine positions (27, 28). Single-stranded DNA from pUC119 *Sac-Sal* was prepared after infection of the transformed *E. coli* CJ236 with an M13 helper phage (K07) (28). Oligonucleotide site-directed mutagenesis was performed (59–61) with the following synthetic DNA oligonucleotides (changed nucleotides are underlined):

5'-CAA TAC TGC GAT GAT TTG-3' (YSDD)
5'-CAA TAC GCG GAT GAT TTG-3' (YADD)
5'-AT CAA TAC GGT GAT GAT TTG TAT GTA-3' (YGDD)
5'-AT CAA TAC CCG GAT GAT TTG TAT GTA-3' (YPDD)
5'-AT CAA TAC GTT GAT GAT TTG TAT GTA-3' (YVDD)

Following mutagenesis, the reaction was stopped by the addition of 0.5 M EDTA (pH 8.0), and the mixture was used to transform competent *E. coli* DH5α. Recombinant plasmids were isolated, and the region containing the mutation was confirmed by the dideoxy technique of Sanger et al. (47) as modified for use with Sequenase (U.S. Biochemicals). By convention, the mutants will be referred to as M184X, where M denotes methionine, 184 denotes the amino acid position in the RT gene, and X denotes the mutant amino acid (Fig. 1B).

To express HIV-1 RT in *E. coli*, a *Bgl*II-*Sal*I restriction

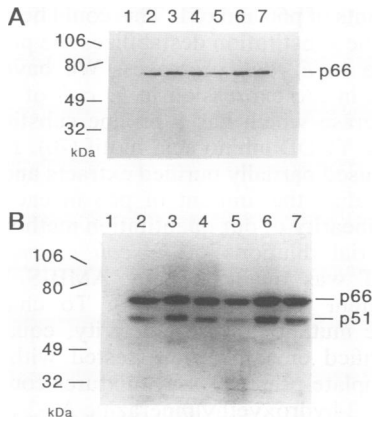

FIG. 2. Expression of HIV-1 wild-type and mutant *pol* genes in *E. coli*. The *Bgl*II-*Sal*I restriction fragment containing the intact HIV-1 protease-RT and integrase genes was subcloned into p*trp*. After expression, extracts were processed and partially purified by phosphocellulose chromatography (20). To analyze expression of HIV-1 RT from *E. coli*, phosphocellulose-purified extracts were electrophoresed on a sodium dodecyl sulfate–10% polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Blots were treated with 50 mM Tris-HCl (pH 7.5)–10 mM NaCl–10% nonfat dry milk (BLOTTO) (22) for 30 min at room temperature to reduce nonspecific binding and incubated overnight with monoclonal antibody to p66 (A) or pooled sera from HIV-1-infected patients (B). After incubation, the blots were washed extensively for 30 min with BLOTTO. Blots reacted with monoclonal anti-p66 were further processed by using the enhanced chemiluminescence system (Amersham), while the blots reacted with the pooled sera from HIV-1-infected patients were reacted with ^{125}I-protein A (100,000 cpm/μg, prepared by using Iodobeads [Bio-Rad] according to the manufacturer's instructions) for 1 h at room temperature. Blots were again washed with BLOTTO for 30 min, air dried, and autoradiographed with Kodak X-Omat film. Lane 1, p*trp*; lane 2, wild type; lane 3, M184A; lane 4, M184G; lane 5, M184P; lane 6, M184S; lane 7, M184V. Molecular mass standards and migration of p66 and p51 forms of the RT are noted.

fragment (nucleotides 2095 through 5819) of the HIV-1 *pol* gene from pUC119 was subcloned into the p*trp* vector, creating an in-frame fusion between the HIV-1 *pol* genes and the *trp* leader peptide (40). In preliminary studies, we have established that expression of the HIV-1 *pol* gene in p*trp* results in the initial synthesis of the polyprotein precursor followed by rapid processing to mature protease, RT, and endonuclease (21). Mature RT is a heterodimer of full-length 66-kDa and COOH-truncated 51-kDa polypeptides (p66/51) (13). The extracts from *E. coli* were partially purified by phosphocellulose chromatography and then analyzed by Western blot (immunoblot) using a monoclonal antibody which reacts with p66 (Fig. 2A) and pooled sera from HIV-1-infected patients (Fig. 2B). Anti-p66 monoclonal antibody detected a single immunoreactive protein with a molecular mass of 66 kDa in extracts transformed with plasmids containing the wild-type or mutant RT genes. No reactivity was detected in extracts from *E. coli* transformed with the p*trp* vector. Two predominant immunoreactive bands with approximate molecular masses of 66 and 51 kDa were observed when the same extracts used for Fig. 2A were reacted with pooled sera from HIV-1-infected patients (Fig. 2B). In several independent experiments, no reproducible differences in the proteolytic processing or p66/51 ratio between the wild-type and mutant RTs were observed. We did note, though, that the mutant M184P consistently had

smaller amounts of p66 and p51. This could be due to the fact that the proline substitution destabilizes the protein, making it susceptible to *E. coli* proteases. We have observed a similar result in the expression in *E. coli* of the poliovirus RNA polymerase which has a proline substitution for the glycine in the YGDD amino acid motif (20). For enzymatic analysis, we used partially purified extracts and the Western blot to normalize the amount of p66 in each extract. To confirm the linearity of our quantitation methods, a Western blot with serial dilutions of *E. coli* extracts containing wild-type RT was quantitated by AMBIS Radioanalytic Imaging System (data not shown). To characterize the effects of the mutations on RT activity, equal amounts of partially purified enzymes were tested with a poly(rA) · oligo(dT) template-primer. Assay mixtures contained 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 8), 2.4 mM magnesium acetate, 10 mM dithiothreitol, 1 μg of poly(A) · oligo(dT), 8 nM TTP, and 2 μCi of [^{32}P]TTP (410 Ci/mmol) (Amersham Co.). For each enzyme, the reaction mixture contained various amounts of extracts in a volume of 50 μl. Reaction mixtures were incubated at 37°C for 30 min unless otherwise noted, and the reactions were terminated by addition of 100 μl of 0.2 M sodium PP_i, 50 μg of tRNA, and 100 μl of 20% trichloroacetic acid. Precipitates were collected by filtration and dried, and radioactivity was determined by scintillation counting. Wild-type RT (M184) demonstrated activity approximately 100-fold greater than that of extracts derived from the vector alone. The M184V, M184A, and M184S mutants exhibited nearly wild-type levels of activity (70 to 100%), while that of mutant M184G was reduced to 5 to 10% of wild-type activity (Fig. 3A). Mutant M184P was completely inactive.

To further characterize the effects of the mutations, we compared the kinetics of in vitro synthesis, Mg^{2+} ion requirements, and temperature sensitivity of the mutant and wild-type enzymes. No reproducible differences between the various enzymes with regard to the kinetics of synthesis were observed (Fig. 3B). We did note that mutant M184G reproducibly exhibited a low level of activity during the extended incubation time. HIV-1 RT requires Mg^{2+} ions for enzymatic activity (46). To determine whether the mutations in the YMDD motif of this enzyme might have significant effects on the enzyme's requirement for Mg^{2+}, we compared the activities of wild-type and mutant enzymes over a range of Mg^{2+} concentrations from 0 to 8 mM. No significant change in the pattern of reactivity for any mutant over the range tested was noted (data not shown). The thermostabilities of the enzymes at 30, 37, 42, and 45°C were determined. Standard reactions were performed with a poly(rA) · oligo(dT) template-primer. The activities of wild-type and mutant enzymes increased as reaction temperature increased from 30 to 42°C, with a slight reduction at 45°C (data not shown).

Since initiation of reverse transcription uses a cellular tRNA as a primer to copy the viral RNA genome, RT has the capacity to recognize both RNA and DNA primers on an RNA template (1, 4, 9, 14, 54, 55). To further test the enzymatic activities of the mutants, we substituted poly(rA) · oligo(U) as the template-primer combination in the standard assay. Previous studies have utilized this template-primer pair to analyze the RNA-dependent RNA polymerase activity of poliovirus (20). In preliminary experiments, we have confirmed that murine leukemia virus RT and avian leukosis virus RT demonstrate considerable in vitro activity with this template-primer (56). Wild-type HIV-1 RT was active on this template-primer, with levels of

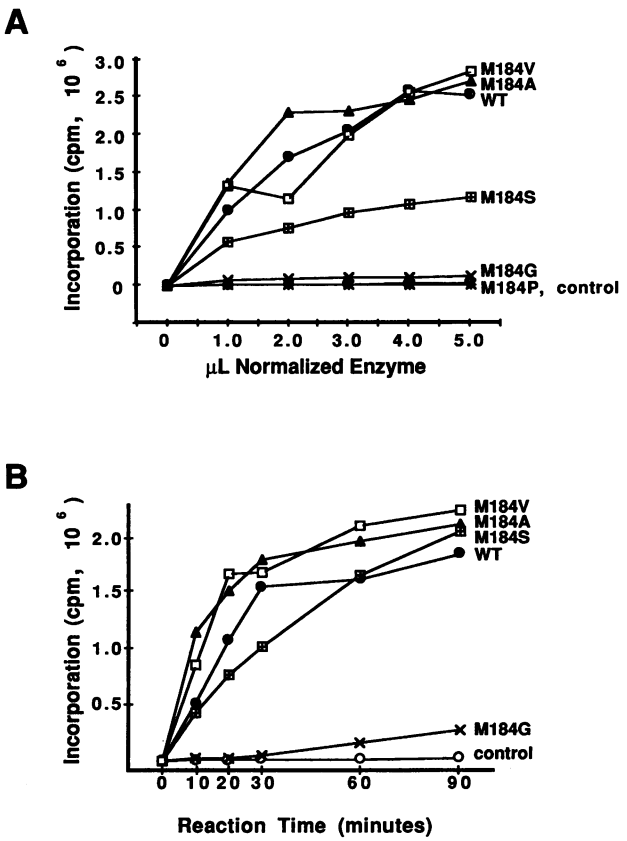

FIG. 3. In vitro enzymatic activities of wild-type and mutant RTs assayed by using a poly(rA) · oligo(dT) template-primer. Each of the extracts was normalized for the levels of immunoreactive p66 determined from the Western blot. (A) Increasing amounts of normalized extracts were analyzed in the in vitro assay, and enzyme activity was determined by incorporation of radioactive TTP. Assays were performed on extracts from *E. coli* transformed with vector, wild-type RT, or mutant RTs by using poly(rA) · oligo(dT) as a template-primer. (B) Kinetics of in vitro synthesis. Wild-type and mutant RTs were analyzed for activity over the indicated time course by using the poly(rA) · oligo(dT) assay with 3 μl of each of the *E. coli* extracts used for panel A. WT, wild type; control, extract from *E. coli* transformed with the *ptrp* vector alone.

incorporation of TTP at least 100-fold greater than that observed with extracts of vector alone. Mutant M184V exhibited activity comparable to that of wild-type RT (Fig. 4). In contrast, M184A and M184S had 5 to 10% of the wild-type activity, while mutants M184G and M184P had no activity above background.

To determine the effects of the mutations on viral replication, each gene was subcloned into pHXB2 *gpt*, which contains an infectious HIV-1 provirus (44, 45). To subclone the mutant RT gene into an infectious HIV-1 proviral genome, a *BclI-SalI* DNA fragment from pUC119 *Sac-Sal* was ligated into pHXB2 *gpt*. Following transformation into *E. coli* HB101 cells, the plasmids from the resulting colonies were screened by restriction digests and confirmed by DNA sequencing (47). In preliminary experiments, recombinant proviruses were transfected into COS-1 cells and analyzed for expression of viral proteins by immunoprecipitation with pooled sera from HIV-1-infected patients. Plasmid DNA containing wild-type or mutant proviral genomes (10 μg) was

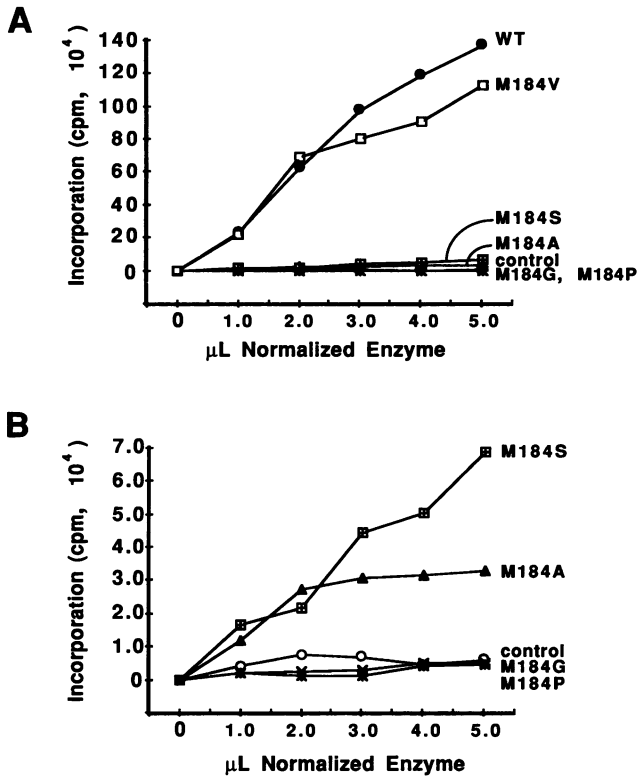

FIG. 4. In vitro enzymatic activities of wild-type and mutant RTs as assayed by using a poly(rA)-oligo(U) template-primer. (A) The assays with wild-type and mutant RTs were performed with normalized amounts of each and poly(rA) · oligo(U) as a template-primer. (B) Replot of the data presented in panel A with an expanded lower scale of incorporation to demonstrate differences between mutant enzymes. WT, wild type; control, extract derived from *E. coli* transformed with p*trp*.

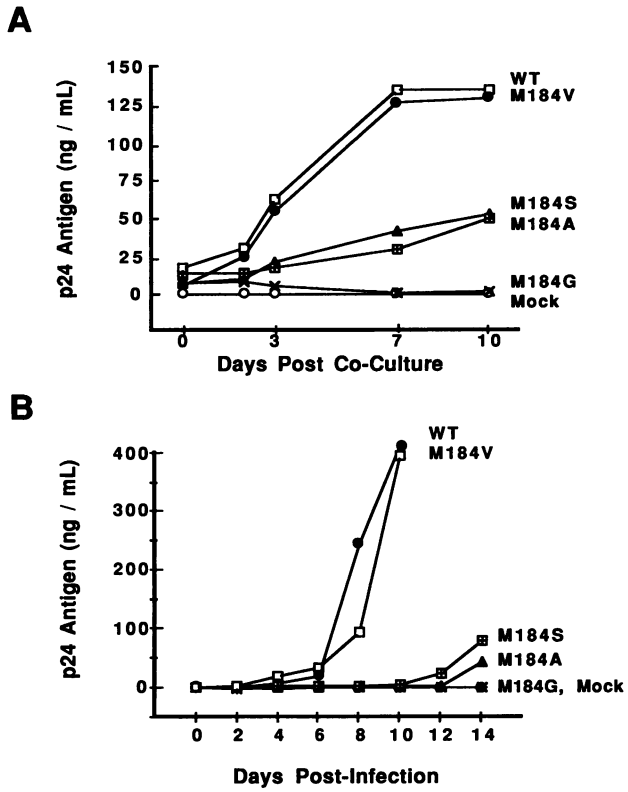

FIG. 5. Replication kinetics of viruses obtained from transfection of proviruses containing mutant RT genes. (A) Coculture infection. Plasmids containing wild-type (pHXB2) or mutant RT genes were transfected into COS-1 cells and 24 h later cocultured with 10^6 SupT1 cells, which support high-level replication of HIV-1. After an additional 24 h, the SupT1 cells were removed by low-speed centrifugation (800 × *g* for 10 min), washed twice in phosphate-buffered saline (PBS), and resuspended in RPMI medium containing 10% fetal bovine serum for further culture. Virus replication was monitored by determination of the amount of p24 antigen in culture supernatants. (B) Cell-free transmission. COS-1 cells were transfected with plasmids containing wild-type (pHXB2) or mutant RT genes. Forty-eight hours posttransfection, supernatants were collected and levels of p24 antigen were determined. The concentration of p24 antigen was then adjusted to 50 ng/ml for each sample and used to infect 10^6 SupT1 cells. Virus was allowed to absorb for 24 h at 37°C. Cultures were washed once with PBS (pH 7.0) and resuspended in RPMI medium containing 10% fetal bovine serum and fresh SupT1 cells (0.5×10^6); culture continued for up to 2 weeks with several passages. Values are in nanograms of p24 antigen per milliliter of culture. WT, wild type; Mock, mock-transfected cultures.

transfected into COS-1 cells by using 300 μg of DEAE-dextran per ml as a facilitator (36). The cells were incubated in DEAE-dextran–DNA for 3 h and then with complete medium (Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum) containing chloroquine (20 μg/ml) for an additional 3 h. A 10% dimethyl sulfoxide shock (1 to 2 min) was added to increase transfection efficiency (36). Cells were washed twice with Dulbecco modified Eagle medium, and then complete medium was added. A similar pattern of HIV-1-specific proteins immunoprecipitated after transfection of the plasmids containing the proviral genomes with the wild-type and mutant RT genes was seen. Both p55 *gag* and p24 as well as *env* proteins (gp120 and gp41) were immunoprecipitated from the cells transfected with the wild type and mutants (data not shown). Thus, the mutations in RT did not affect overall expression of viral proteins.

The infectivity of proviruses containing the RT mutations was next examined. Since COS-1 cells do not support HIV-1 replication, the transfected cells were cocultured with SupT1 cells, which support high-level replication of HIV-1 virus, for 3 days. SupT1 cells were isolated by centrifugation and further cultured with new media and fresh SupT1 cells. Samples of the culture supernatant were removed and assayed for the presence of p24 antigen (Coulter Laboratories) as well as inspected for multinucleated cells (syncytia). We used the analysis of supernatant p24 antigen to reflect virus replication because of the different activities of the wild-type and mutant RTs in the in vitro reactions. The levels of p24 antigen in cultures arising from transfection of a proviral genome containing the M184V mutation were similar to that of the wild type. Proviruses with M184A or M184S mutations gave rise to virus, although the kinetics of appearance were slower and overall levels of p24 antigen in the culture supernatant were less than that for the wild-type virus. Proviruses with M184G or M184P were noninfectious over the culture period (Fig. 5A).

In a second set of experiments, we analyzed the replication kinetics using cell-free virus transmission. Forty-eight hours after transfection of COS-1 cells with the mutant viral

genomes, supernatants were removed and clarified by low-speed centrifugation and the amount of p24 antigen was quantitated. Cultures of fresh SupT1 cells were infected with virus-containing supernatant normalized to 50 ng of p24 antigen per ml. After adsorption and washing to remove excess virus, the cultures were refed and monitored for virus production (Fig. 5B). Replication of the different viruses again paralleled that observed in the coculture experiments. Viruses derived from transfection of proviruses with the wild type and M184V grew rapidly and spread throughout the culture so that by 6 to 8 days, syncytia were evident, with high levels of p24 antigen in the culture supernatant. Viruses derived from transfection of proviruses with M184A and M184S exhibited considerably slower kinetics of infection. With extended culture times, replication of the viruses derived from proviruses with the M184A and M184S was evident. Under the conditions for cell-free transmission, no virus from cells transfected with proviral genomes with the M184G or M184P RT mutation was detected.

The conserved YXDD motif is found in numerous viral RTs, RNA polymerases, and RT-containing elements (2, 11, 19, 25, 29, 30, 43, 48, 50). A similar sequence motif, YGDTDS, is also found in many DNA-dependent DNA polymerases (57). Previous studies have described polymerases in which the first aspartic acid of this conserved motif was mutated (33, 34, 37); in all cases, the enzymes were inactive, supporting the idea that the YXDD motif is involved in the catalytic function. In particular, the aspartic acids have been proposed to bind divalent metal ions which promote a phosphoryl transfer reaction (6, 26). Computer modeling studies have also predicted an important function for the conserved YXDD or YGDTDS motif. On the basis of the three-dimensional structure of Klenow fragment of *E. coli* DNA polymerase I (41), Delarue et al. (10) and Haffey et al. (16) have described hypothetical structural models of the catalytic domain of polymerase α-like enzymes in which the YGDTDS motif constitutes a connecting loop of a β-hairpin structure analogous to the β-hairpin structure that delineated strands 12 and 13 of the Klenow fragment of *E. coli* DNA polymerase I. A similar β-hairpin structure for the YMDD motif has been observed in the recently described three-dimensional structure of HIV-1 RT (26).

Although mutations of the aspartic acids resulted in enzymes with drastically reduced activities, mutations of the second, least-conserved residues of YXDD and YGDTDS motifs of various polymerases have produced enzymes with various levels of activities (7, 12, 18, 20, 24, 37, 39). The YMDD motif is conserved in RTs from HIV-1, HIV-2, human T-cell leukemia virus type I (HTLV-I), HTLV-II, and Rous sarcoma virus. However, RTs from murine leukemia virus and feline leukemia virus have a YVDD amino acid motif. Our in vitro assays demonstrate that the M184V change in HIV-1 RT results in an enzyme indistinguishable from the wild type, and when introduced into an infectious clone it displayed similar virus replication kinetics. Thus, our results imply that there is enough similarity within the YXDD region between these RTs that methionine can be substituted for a valine without deleterious effects on enzyme function. It was clear from the results that the M184A and M184S mutations in the HIV-1 RT resulted in enzymes with in vitro properties similar to those of the wild-type enzyme with a poly(rA) · oligo(dT) primer-template combination. The fact that alanine can substitute for methionine is interesting because a YADD motif has been found in RTs associated with *M. xanthus* (19, 29) and *E. coli* (30). Although these prokaryotic RTs have functions similar to those of their viral counterparts, they are positioned on different branches of the proposed evolutionary tree, and thus it was speculated that they diverged early from the retrovirus-encoded RTs (58). Mutant M184G was chosen because numerous RNA-dependent RNA polymerases, as typified by poliovirus and Qβ bacteriophage, contain a YGDD motif. However, mutant M184G drastically affected the HIV-1 RT, resulting in 5 to 10% of the in vitro activity of the wild type. From the three-dimensional structure, it is clear that there exist potential interactions between the YXDD motif and surrounding amino acids which could be effected by the amino acid changes in this region (26).

Although numerous studies have described the effects of mutations on in vitro activities of RT, few studies have investigated effects of mutations on virus replication. It was suggested that an in vitro RT activity greater than 70% of the wild type on a poly(A) · oligo(dT) template-primer was necessary for production of infectious virus (32). These results are in partial agreement with ours, in that transfection of proviruses with the M184V, M184S, and M184A mutations, which had enzyme activities comparable to that of the wild type using a poly(rA) · oligo(dT) primer-template, consistently yielded infectious virus. However, it was interesting that the capacities of wild-type and mutant RTs to utilize a poly(rA) · oligo(U) template-primer correlated with the infectivities of proviruses containing mutant RT genes. Initiation of the reverse transcription of retroviral genomes utilizes a cellular tRNA as a primer to copy the viral RNA genome. HIV-1 RT thus has the capacity to recognize an RNA template-primer. Mutants M184S and M184A demonstrated 5 to 10% of the in vitro activity of the wild type or mutant M184V on a poly(rA) · oligo(U) template-primer. Furthermore, proviruses with the M184A or M184S mutation gave rise to viruses which demonstrated slower replication than viruses from the wild type or the M184V mutant. This result suggests that proviruses with the M184A or M184S mutation in the RT gene might give rise to viruses that are inefficient in initiation of reverse transcription. To test this possibility, experiments are under way to examine the early events after infection with viruses containing the wild-type or M184S or M184A mutant RT gene.

In conclusion, these results point to the critical structural role that the YMDD motif plays in the enzymatic activity of RT and highlight the fact that subtle mutations of methionine drastically affect the activity and subsequent replication potential of the virus.

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