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The Role of Steric Hindrance in 3TC Resistance of Human Immunodeficiency Virus Type-1 Reverse Transcriptase

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Treating HIV infections with drugs that block viral replication selects for drug-resistant strains of the virus. Particular inhibitors select characteristic resistance mutations. In the case of the nucleoside analogs 3TC and FTC, resistant viruses are selected with mutations at amino acid residue 184 of reverse transcriptase (RT). The initial change is usually to M184I; this virus is rapidly replaced by a variant carrying the mutation M184V. 3TC and FTC are taken up by cells and converted into 3TCTP and FTCTP. The triphosphate forms of these nucleoside analogs are incorporated into DNA by HIV-1 RT and act as chain terminators. Both of the mutations, M184I and M184V, provide very high levels of resistance *in vivo*; purified HIV-1 RT carrying M184V and M184I also shows resistance to 3TCTP and FTCTP in *in vitro* polymerase assays. Amino acid M184 is part of the dNTP binding site of HIV-1 RT. Structural studies suggest that the mechanism of resistance of HIV-1 RTs carrying the M184V or M184I mutation involves steric hindrance, which could either completely block the binding of 3TCTP and FTCTP or allow binding of these nucleoside triphosphate molecules but only in a configuration that would prevent incorporation. The available kinetic data are ambiguous: one group has reported that the primary effect of the mutations is at the level of 3TCTP binding; another, at the level of incorporation. We have approached this problem using assays that monitor the ability of HIV-1 RT to undergo a conformational change upon binding a dNTP. These studies show that both wild-type RT and the drug-resistant variants can bind 3TCTP at the polymerase active site; however, the binding to M184V and M184I is somewhat weaker and is sensitive to salt. We propose that the drug-resistant variants bind 3TCTP in a strained configuration that is salt-sensitive and is not catalytically competent.

Keywords: HIV-1, reverse transcriptase, 3TC, drug resistance, RNase H

Introduction

The viral enzymes reverse transcriptase (RT) and protease (PR) are the targets for the drugs used to treat individuals infected with human immunodeficiency virus type-1 (HIV-1), the causative agent for AIDS. However, drug treatment often selects for drug-resistant variants of the virus that have mutations in the target protein. There are two classes of RT inhibitors, nucleoside analogs and non-nucleoside inhibitors. Nucleoside analog RT inhibitors lack the 3′OH of the ribose ring. When taken up by cells, nucleoside analogs are converted into nucleoside (analog) triphosphate groups, which, when incorporated into viral DNA by HIV-1 RT, cause termination of the DNA chain. 3TC and FTC are both nucleoside analogs. 3TC treatment of HIV-1 infected patients selects for

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Abbreviations used: RT, reverse transcriptase; PR, protease; HIV-1, human immunodeficiency virus type 1; dsDNA, double-stranded DNA; BSA, bovine serum albumin.

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drug-resistant variants with changes at position 184. Usually the variant M184I appears first, then is replaced by M184V. Viruses carrying either of these mutations are very resistant to both 3TC and FTC; purified HIV-1 RT carrying either mutation is resistant to inhibition by 3TCTP or FTCTP in *in vitro* polymerase assays.

Structural analyses have provided models to explain the high level of resistance of the M184I and M184V mutants for 3TC and FTC. 3TC and FTC have the ribose ring replaced by an oxathiolane ring; the stereochemical form of 3TC and FTC which are used to treat HIV-1 infections is the opposite enantiomer relative to normal dNTPs. The combination of introducing a sulfur atom into the ribose ring and choosing the opposite enantiomer causes the portion of the oxathiolane ring that carries the sulfur atom to project farther than the normal ribose ring, thus creating an opportunity for steric hindrance (Huang *et al*., 1998; Sarafianos *et al*., 1999). Because the wild-type enzyme readily incorporates 3TCTP and FTCTP, there can be no significant steric hindrance with M184. However, models developed based either on a ternary complex of wild-type HIV-1 RT, double-stranded DNA (dsDNA) and a bound dNTP (Huang *et al*., 1998) or on the structure of the M184I mutant bound to dsDNA (Sarafianos *et al*., 1999) suggest that a β-branched amino acid at position 184 would interfere with the ability of either 3TCTP or FTCTP to bind in the appropriate configuration at the polymerase active site. This mechanism not only accounts for the resistance of the M184I and M184V variants of HIV-1 RT to 3TC and FTC, but also explains the resistance to 3TC of variants of feline immunodeficiency virus that have another β-branched amino acid residue (threonine) at a position equivalent to 184 of HIV-1 RT. This same model can also explain the resistance of hepatitis B virus RT with either valine or isoleucine at the position equivalent to 184 of HIV-1 RT (Sarafianos *et al*., 1999).

The models do not answer the question of exactly how the steric hindrance manifests itself for the M184I and M184V variants. It is possible that the steric hindrance blocks the binding of the incoming 3TCTP or FTCTP altogether, or that binding occurs, but only in a configuration that interferes with incorporation. Three groups have used kinetic analysis to investigate either FTCTP or 3TCTP binding and incorporation by the M184V variant; however, these groups have not reached the same conclusions. Krebs *et al*. (1997) proposed that, relative to wild-type HIV-1 RT, the binding of 3TCTP to M184V is minimally affected, but that there is a significant effect on the rate of incorporation. Feng & Anderson (1999) reported the opposite result: the primary effect of M184V is on binding, not incorporation. Wilson *et al*. (1996) using FTCTP, also reported that the primary effect is on binding, not incorporation. Given the similarities in these two inhibitors, it seems unlikely that the mechanisms of resistance are radically different.

To try to resolve this issue, we have taken a different approach. The binding of a dNTP causes a specific change in the structure of HIV-1 RT; when a dNTP binds at the polymerase active site, the fingers of RT close onto the dNTP, creating the dNTP-binding pocket (Huang *et al*., 1998). Other DNA polymerases (T7 DNA polymerase and Klen-taq polymerase) undergo related structural changes upon dNTP binding (Doublie *et al*., 1998; Li *et al*., 1998). Normally, the closed configuration is transient, and immediately precedes the incorporation of the incoming dNTP. However, if the nucleoside at the 3′-end of the primer strand does not have a 3′OH group, a stable dead-end complex is formed. This closed HIV-1 RT complex can be detected by gel electrophoresis (Tong *et al*., 1997) and has been studied crystallographically (Huang *et al*., 1998). We show here that this closed complex is associated with a characteristic change in the specificity of RNase H cleavage. The gel shift and the RNase H assays can be used to monitor the ability of wild-type and mutant HIV-1 RT to form the closed complex with either normal dNTPs or nucleoside analogs. Using these assays, we measured the ability of both 3TCTP and dCTP to induce the closed configuration for wild-type and mutant HIV-1 RT. As expected, wild-type HIV-1 RT can form a closed complex with both 3TCTP and dCTP. Both of the 3TC resistant mutants, M184V and M184I, will also form complexes with dCTP and 3TCTP; however, in the case of the mutants, but not the wild-type RTs, the 3TCTP complex is salt-sensitive. This suggests, although a closed complex can be formed with the mutant enzymes and that the complex is strained, presumably by steric hindrance, that this strained configuration is not catalytically competent.

Results

Structure of the RT/nucleic acid complex

Crystallographic analyses have shown that the preferred mode of binding nucleic acid by HIV-1 RT places the 3′-end of the primer strand at the polymerase active site (Jacobo-Molina *et al*., 1991; Ding *et al*., 1998; Huang *et al*., 1998). The RNase H active site lies opposite the template strand approximately 17 base-pairs from the polymerase active site. This configuration corresponds to the initial cleavages that RT makes with most RNA/DNA duplexes; the initial cleavages center around a position about 17 base-pairs from the 3′-end of the DNA primer. Longer incubation produces additional cleavages that center around positions about eight base-pairs from the polymerase active site (Schatz *et al*., 1990; Fu & Taylor, 1992; Gopalakrishnan *et al*., 1992; Peliska & Benkovic, 1992; Cirino *et al*., 1995; Ghosh *et al*., 1995; Götte *et al*., 1995; Gao *et al*., 1998). For the −8 cleavages to occur, the relationship between HIV-1 RT and

the nucleic acid substrate must undergo a dramatic change. Although the exact nature of this change is not known, it seems unlikely that an enzyme with a bound dNTP in the closed configuration could undergo the structural changes necessary to permit the −8 cleavage.

The site of −17 cleavage is altered in the closed complex, and the −8 cleavage is blocked

To test the idea that the formation of the closed complex affects RNase H cleavage, we used an 81-base RNA and a 20-base DNA oligonucleotide (Gao *et al.*, 1998). With this substrate, HIV-1 RT makes the characteristic −17 and −8 cleavages (Figure 1). To facilitate the preparation of a stable form of the closed complex, we prepared DNA oligonucleotides (both a 19-mer and a 20-mer) that had a dideoxy nucleoside at their 3′-ends, and the corresponding 19-mer and 20-mer DNA oligonucleotides with normal nucleosides at their 3′-ends (see Materials and Methods). The only difference in the sequences of the 19-mer and 20-mer DNA oligonucleotides is at their 3′-ends: The 19-mer is missing the 3′ most base of the 20-mer. The dideoxy-terminated DNA oligonucleotides were used to prepare closed complexes with the 81-base RNA template, HIV-1 RT, and appropriate dNTPs.

In the absence of an added triphosphate group, the cleavage patterns of the duplexes made with the two 19-mers (both the fully normal oligonucleotide and the version with a dideoxy nucleoside at the 3′-end were quite similar; the cleavage patterns of the duplexes made with the two 20-mers were also very similar. However, when substrates prepared with either the dideoxy blocked 19-mer or 20-mer were incubated with HIV-1 RT and the dNTP that would be bound at the polymerase active site, the position of the −17 cleavages was

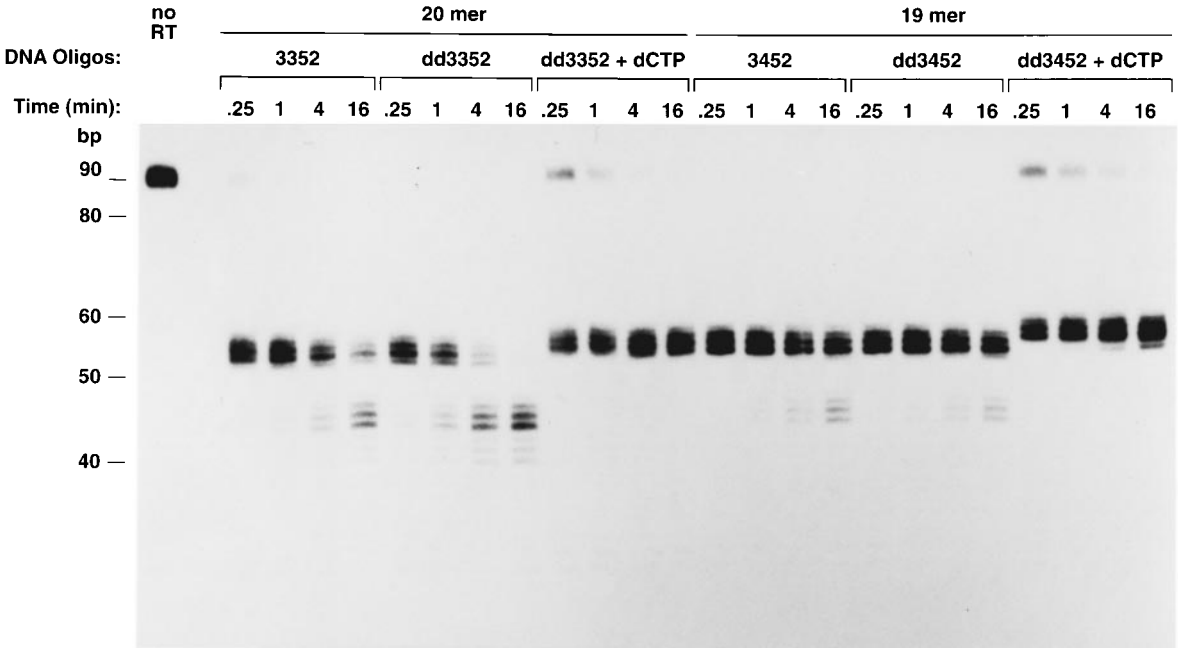

Figure 1. Effects of binding the cognate dNTP to the polymerase active site of HIV-1 RT on RNase H activity. RNase H assays were performed with a radioactively labeled 81-base RNA derived from the genome of HIV-1 (Gao *et al.*, 1998). This RNA was hybridized with one of the four related DNA oligonucleotides that either have a normal 3′ nucleotide or a dideoxy 3′ nucleotide. The oligonucleotides are either 19 or 20 bases long and bind to positions (on the RNA) 34 to 52 (3452 and dd3452) or 33 to 52 (3352 and dd3352). Incubation times were varied between 0.25 minute and 16 minutes for each sample. The digests were fractionated on a 15% polyacrylamide sequencing gel; the RNA fragments were detected by autoradiography. The sequences of the RNA template and the complementary DNA oligonucleotide were chosen so that, after RNase H digestion, all of label remains in the 5′ fragment simplifying the interpretation of the data (Gao *et al.*, 1998). The migration of DNA markers was used to gauge the sizes of the products of RNA cleavage; although the sizes of the DNA markers and the RNA cleavage products do not correspond directly, we have calibrated their relative migration (Gao *et al.*, 1998). When HIV-1 binds to nucleic acid, the polymerase active site is at the 3′-end of the primer, the RNase H active site is aligned with the template strand approximately 17 bases 3′ (on the template strand). This corresponds to the initial sites of RNase H cleavage, approximately 17 bases from the 3′-end of the primer. These produce RNA fragments that migrate on the gel between the 50-base and 60-base DNA oligonucleotide markers. In the absence of a cognate dNTP (final concentration 300 μM), longer incubation produced cleavages approximately −8 from the 3′-end of the primer. These are the RNA bands that migrate at positions between the 40-base and 50-base DNA markers. Note that the addition of the cognate base altered the position of the primary cleavage (compare dd3452 and dd3452 + dCTP in the last eight lanes on the right).

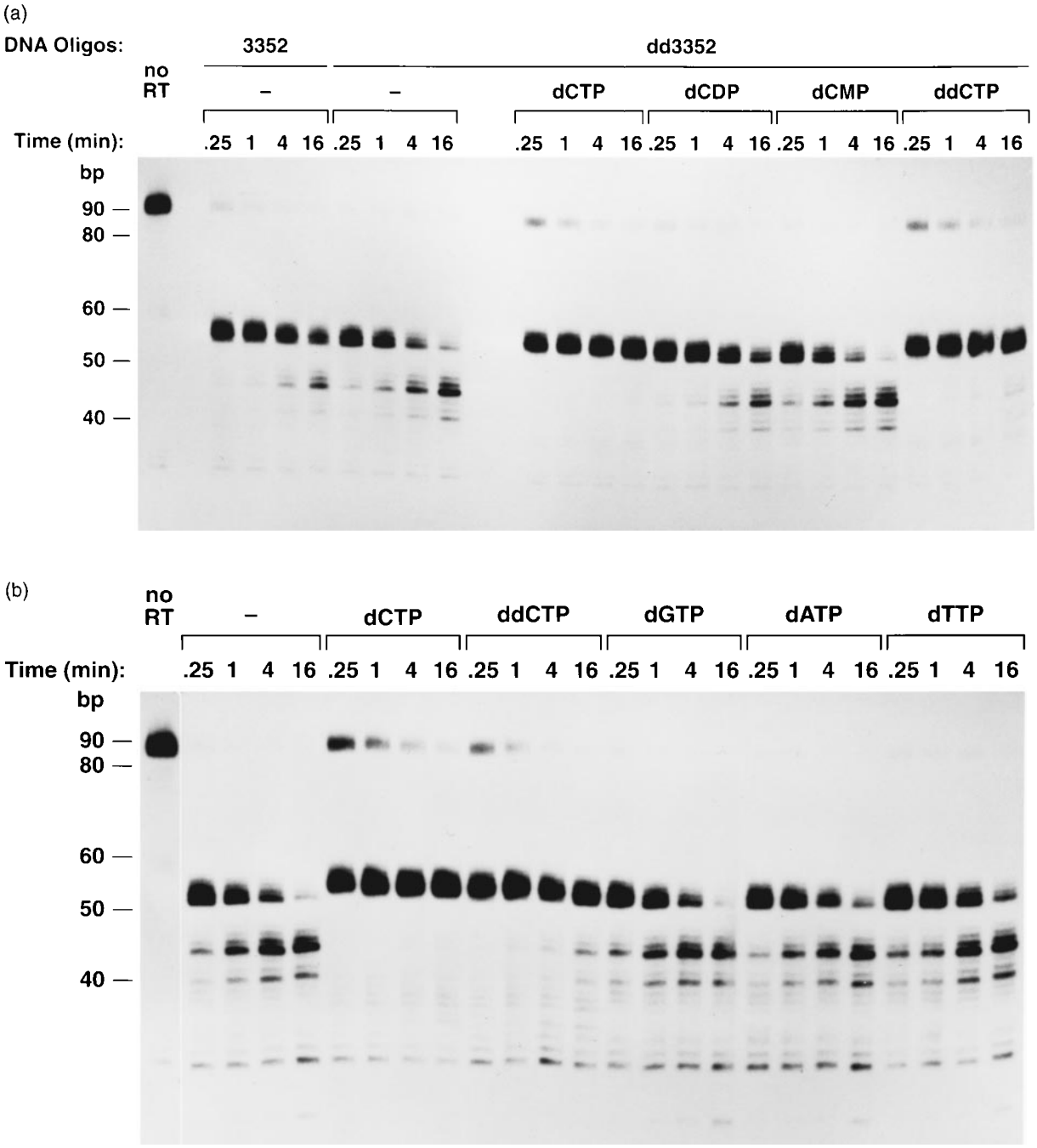

Figure 2. Effects of other nucleosides on RNase H cleavage. RNase H assays and sample analysis were done as described in the legend to Figure 1 and in Materials and Methods. (a) Effects of adding the cognate nucleoside (mono-, di- and triphosphate). RNase H digests were done with the substrate created with the dideoxy terminated 20-base DNA oligonucleotide (dd3352) in the presence of dCTP, dCDP, dCMP, and ddCTP (see Materials and Methods). (b) Effects of adding non-cognate nucleoside triphosphates.

altered and the −8 cleavages were diminished or eliminated (see Figures 1 and 2). The alteration of the specificity of the −17 cleavages can be clearly seen in the last eight lanes of Figure 1.

We believe that these changes in RNase H cleavage occurred because the dNTP was bound at the polymerase active site, causing a change in the conformation of HIV-1 RT that affects the pattern of RNase H cleavage. The loss of the −8 cleavages is easily explained; the closed complex is a relatively stable structure, and presumably prevents the changes in the positioning of the nucleic acid substrate relative to the RNase H active site that are required for the −8 cleavages. Although we were not surprised to see a change in the position of the −17 cleavages, the type of change that was seen was unexpected. Rather than causing the cleavages to occur closer to the 5′-end of the RNA, the

addition of the dNTP moved the −17 cleavages approximately one base closer to the 3′-end of the RNA. This phenomenon was seen with substrates prepared with either the dideoxy terminated 19-mer or the 20-mer DNA oligonucleotides, suggesting that the change in relative position of the −17 cleavages was not a special property of one particular position on the substrate but was related to conformational changes in the RT nucleic acid complex. The fact that, in the closed complex, RT still makes multiple cleavages near −17 also has implications for the flexibility of the enzyme (see Discussion).

In these assays, relatively high concentrations of dNTP were required to produce a complete block of the −8 cleavage. Because the cleavage assay was carried out for periods of up to 16 minutes, we believe that a relatively high concentration of dNTP was required to keep the complex in the closed position for the entire assay period (Figure 1).

Blocking the −8 cleavage requires the cognate dNTP

If, as we have proposed, the changes in the pattern of RNase H cleavage are caused by HIV-1 RT adopting the closed configuration, then there should be a specific requirement for the cognate dNTP. This conjecture was tested in several ways. First, the three dNTPs that would not appropriately base-pair with the template strand at the polymerase active site were tested. The non-cognate dNTPs did not affect RNase H cleavage or produce a shift in the position of the −17 cleavages. Second, the monophosphate and diphosphate forms of the cognate dNTP were tested. The monophosphate form had no detectable effect on RNase H cleavage; the diphosphate had a small, but measurable, effect. Third, the dideoxy form of the cognate dNTP was tested. It yielded a pattern of RNase H cleavage similar to that caused by the normal dNTP (Figure 2). Taken together, these data show that exactly those nucleosides that would be able to bind at the polymerase active site and induce the closed conformation of HIV-1 RT are those that affect the pattern of RNase H cleavage. These observations provide strong support for the idea that the alteration in the pattern of RNase H cleavage can be used to monitor the change in the structure of HIV-1 RT that accompanies the binding of a dNTP at the polymerase active site.

Properties of the HIV-1 RT mutants M184V and M184I

Although wild-type HIV-1 RT and the M184V and M184I mutants have been studied extensively, both by our laboratory and by others, we wanted to be certain that the wild-type and mutant enzymes had the appropriate sensitivity/resistance to inhibition by 3TCTP in an *in vitro* polymerase assay. As expected, both mutant enzymes M184V and M184I were substantially more resistant to 3TCTP than was wild-type HIV-1 RT (data not shown).

Several laboratories, including ours, have reported that HIV-1 RTs with amino acid residue substitutions at position 184 are less processive than the wild-type enzyme (Boyer & Hughes, 1995; Avidan & Hizi, 1998; Quan *et al*., 1998; Sharma & Crumpacker, 1999). We also tested the ability of the RTs to extend a labeled primer in the presence of relatively low concentrations of dNTPs. In the extension assays, at any given dNTP concentration, both of the RTs with amino acid substitutions at position 184 made cDNAs that were, on average, smaller than the cDNAs made by the wild-type enzyme. The M184V variant is slightly better at making longer cDNA products than is the M184I variant. This result is consistent with the previous data obtained with processivity assays performed at a higher concentration of dNTPs (data not shown).

Effect of dCTP and 3TCTP on the RNase H activity of wild-type and mutant HIV-1 RT

The extension assays performed at limiting dNTP concentrations showed that the ability of the mutant enzymes to bind and incorporate normal dNTPs was only slightly reduced relative to wild-type HIV-1 RT (data not shown). We tested both the ability of the mutant enzymes to make the −17 and −8 cleavages and the ability of the cognate dNTP (dCTP) to alter the −17 cleavages and block the −8 cleavages. As shown in Figure 3, both of the mutant enzymes behaved similarly to wild-type HIV-1 RT.

We also tested the ability of 3TCTP to block the −8 cleavage. The RNase H cleavage specificity of all three enzymes was profoundly affected by the addition of high concentrations of 3TCTP (Figure 3). We attribute this result to a change in the configuration of the enzyme, suggesting that 3TCTP can bind to all the enzymes, wild-type and the two 184 mutants, in a way that induces the closed conformation. However, higher concentrations of 3TCTP were required to block the ability of the mutant enzymes to make the −8 cleavage (Figure 3) than were required to block the −8 cleavage by for wild-type HIV-1 RT. For M184I this effect is most easily seen by looking at the effect on the −17 cleavages (compare Figure 3(c) and (f) at the lowest concentrations of dCTP and 3TCTP). This result suggests that, although the mutant enzymes can bind 3TCTP in a closed conformation, the process is not as efficient as it is for the wild-type enzyme.

Gel shift assays

It has been reported that the closed form of HIV-1 RT induced by binding a dNTP can be detected using a gel shift assay (Tong *et al*., 1997). This assay

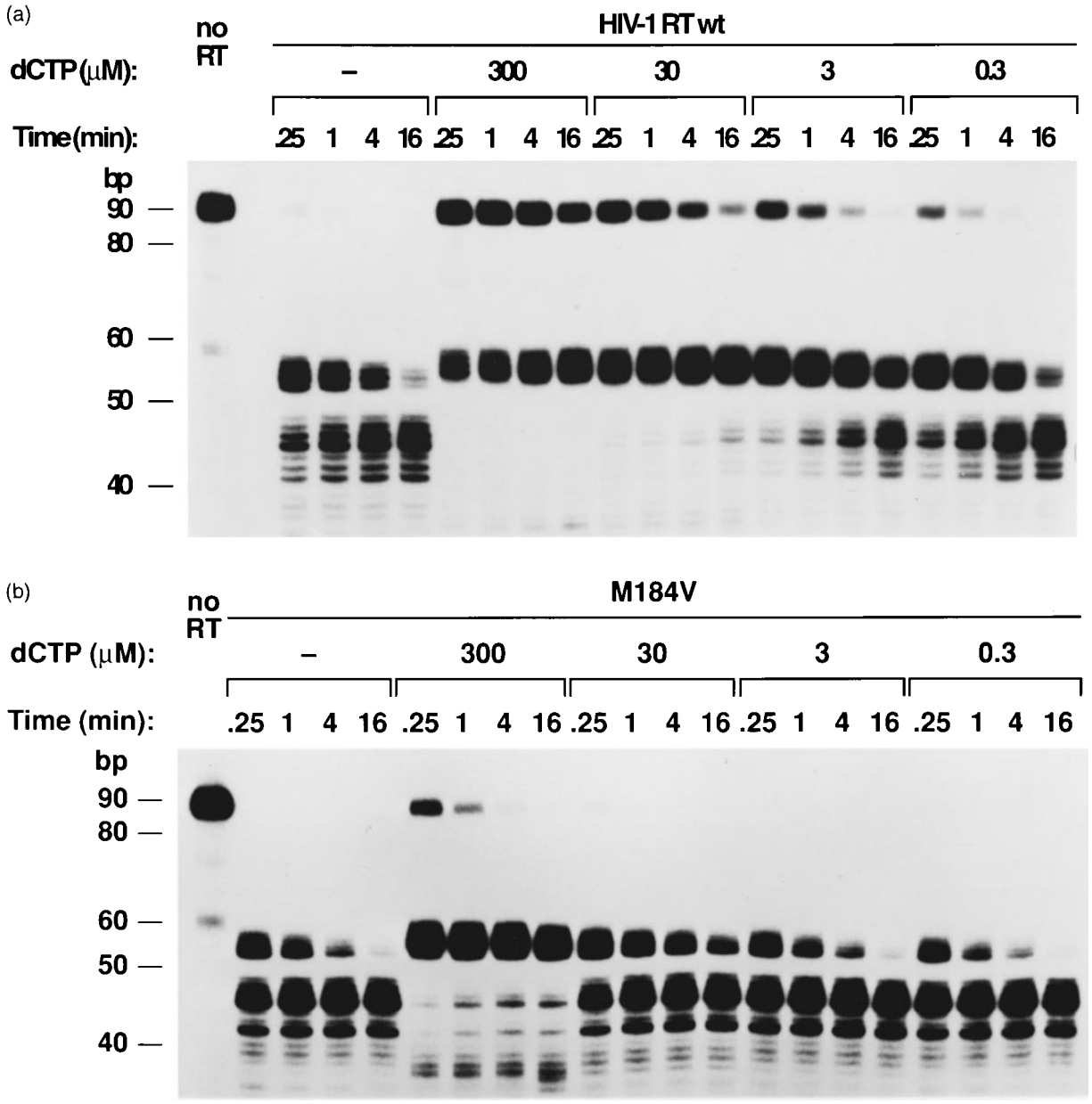

Figure 3 (*legend shown on page 410*)

depends on the fact that the closed complex that contains a dNTP is more stable than the binary complex formed when HIV-1 RT binds DNA. We initially performed the assay under the conditions described by Tong *et al.* (1997). However, in our experiments, the binary complex was relatively stable under the conditions described by Tong *et al.* (1997) and we were not able to distinguish the closed (tertiary) complex from the binary complex (data not shown). However, we found that the two complexes can be distinguished in the presence of higher salt concentrations. If the complexes were allowed to form in low salt concentrations and then incubated in a higher salt buffer (see Materials and Methods), the closed ternary complex can be detected specifically in the gel shift assay (Figure 4).

We first compared the ability of dCTP to form closed complexes with wild-type HIV-1 RT and the M184V mutant. Although the M184V required somewhat higher concentrations of dCTP, both enzymes were able to form closed complexes. The fact that the M184V mutant requires slightly higher concentrations is in agreement with data obtained in the processivity assay; it appears that the wild-type enzyme has a slightly higher affinity for dNTPs than does M184V (Figure 4).

The ability of the two enzymes, wild-type HIV-1 RT and M184V, to form a closed complex with

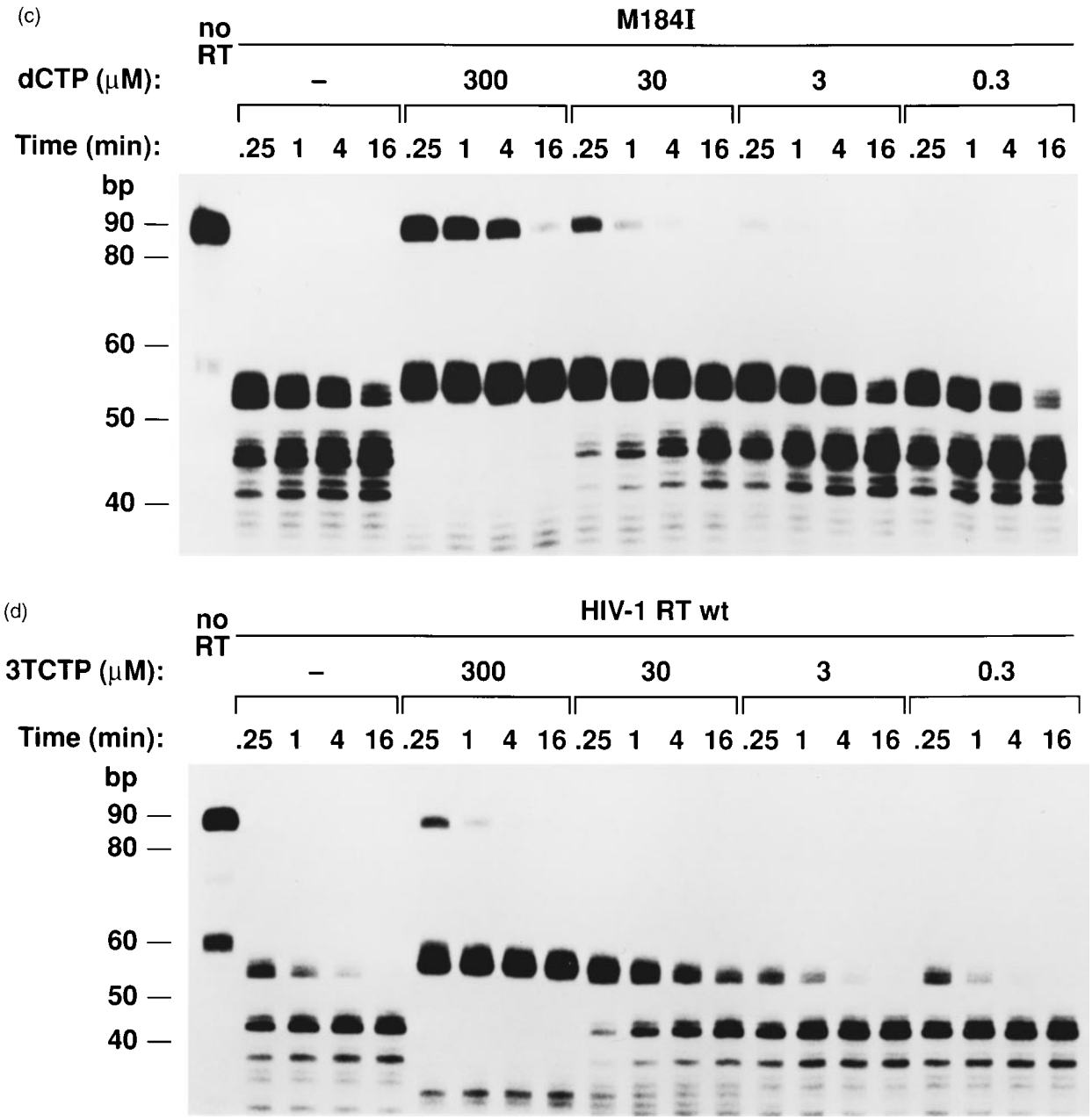

Figure 3 (*legend shown on page 410*)

3TCTP was then measured using the gel shift assay. These data show that the wild-type enzyme binds 3TCTP less well than dCTP. Under the conditions we used, the M184V mutant did not form a stable closed complex that can be detected using the gel shift assay even if high levels of 3TCTP were used (Figure 4). Because we detected a closed complex with the M184V mutant and 3TCTP in the RNase H assay, we knew that a closed complex could be formed at low salt concentrations. In the gel shift assay, a complex was allowed to form in low salt concentrations, then the salt concentration was raised. A simple interpretation of the data is that, for the M184V mutant the 3TCTP complex is strained, and the combination of the higher salt and the fractionation on the gel caused the closed complex to dissociate.

This interpretation of the data would predict that, if the RNase H assay were done in the higher salt conditions used for the gel shift assay, 3TCTP would be able to block the −8 cleavage for the wild-type enzyme, but not for the M184V mutant. We repeated the RNase H assay using the salt conditions similar to those used in the gel shift assay. The results were as we predicted: at the higher salt conditions, 3TCTP blocked the ability of the wild-type enzyme to make the −8 cleavages. In this assay, slightly higher concentrations of dCTP

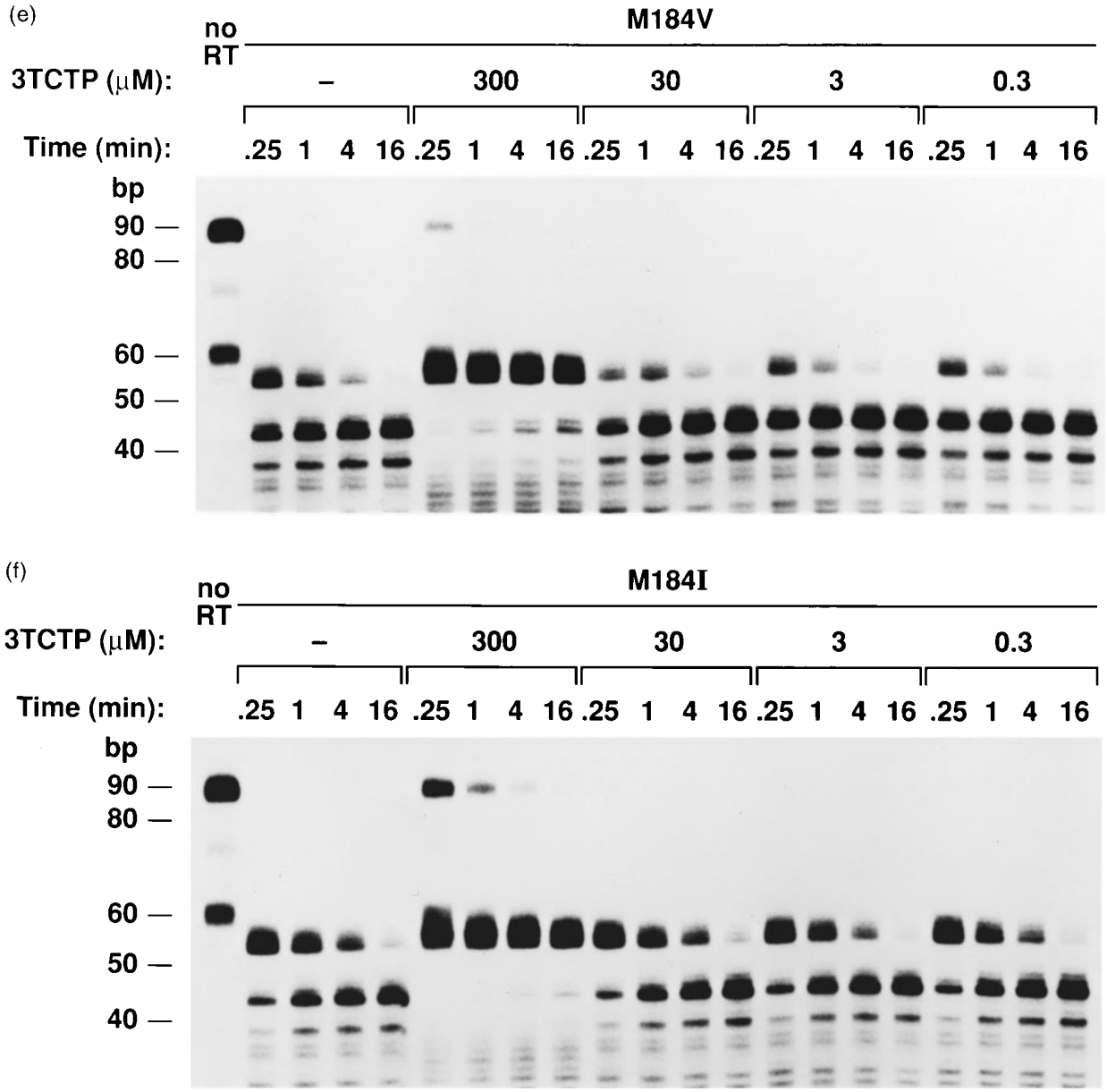

Figure 3. Effects of various concentrations of dCTP and 3TCTP on RNase H cleavage. The RNase H substrate was prepared with the dideoxy terminated 20-base oligonucleotide (dd3352) and the RNase H reactions were performed in the presence of various concentrations (0.3-300 μM) of dCTP or 3TCTP. The reactions were done and the cleavage products were analyzed as described in Materials and Methods and in the legend to Figure 1. (a)-(c) RNase H reactions were performed with wild-type RT or M184V or M184I in the presence of dCTP. (d)-(f) RNase H reactions were performed with wild-type HIV-1 RT or M184V or M184I in the presence of 3TCTP.

appear to be required to block the −8 cleavage by wild-type HIV-l RT (compare Figures 3(a) and 5(a)). However; under the higher salt conditions, 3TCTP cannot block the −8 cleavages made by M184V (Figure 5). This is not simply an effect on the ability of any dNTP to bind; dCTP still can block the −8 cleavage for both wild-type HIV-1 RT and the M184V mutant. We also performed the gel shift assay and the high-salt version of the RNase H assay on the M184I mutant. The results obtained with M184I were quite similar to those obtained with M184V (data not shown).

Discussion

The M184V and M184I mutations confer high-level resistance to 3TC and FTC. This resistance can be demonstrated in *in vitro* polymerase assays performed with 3TCTP and FTCTP. Models developed based on the structures of a ternary complex of wild-type HIV-1 RT, DNA, and a dNTP and of a binary complex of M184I and DNA suggest that resistance is based on steric hindrance. In these models, the oxathiolane ring of 3TCTP and FTCTP makes unfavorably close contact with the

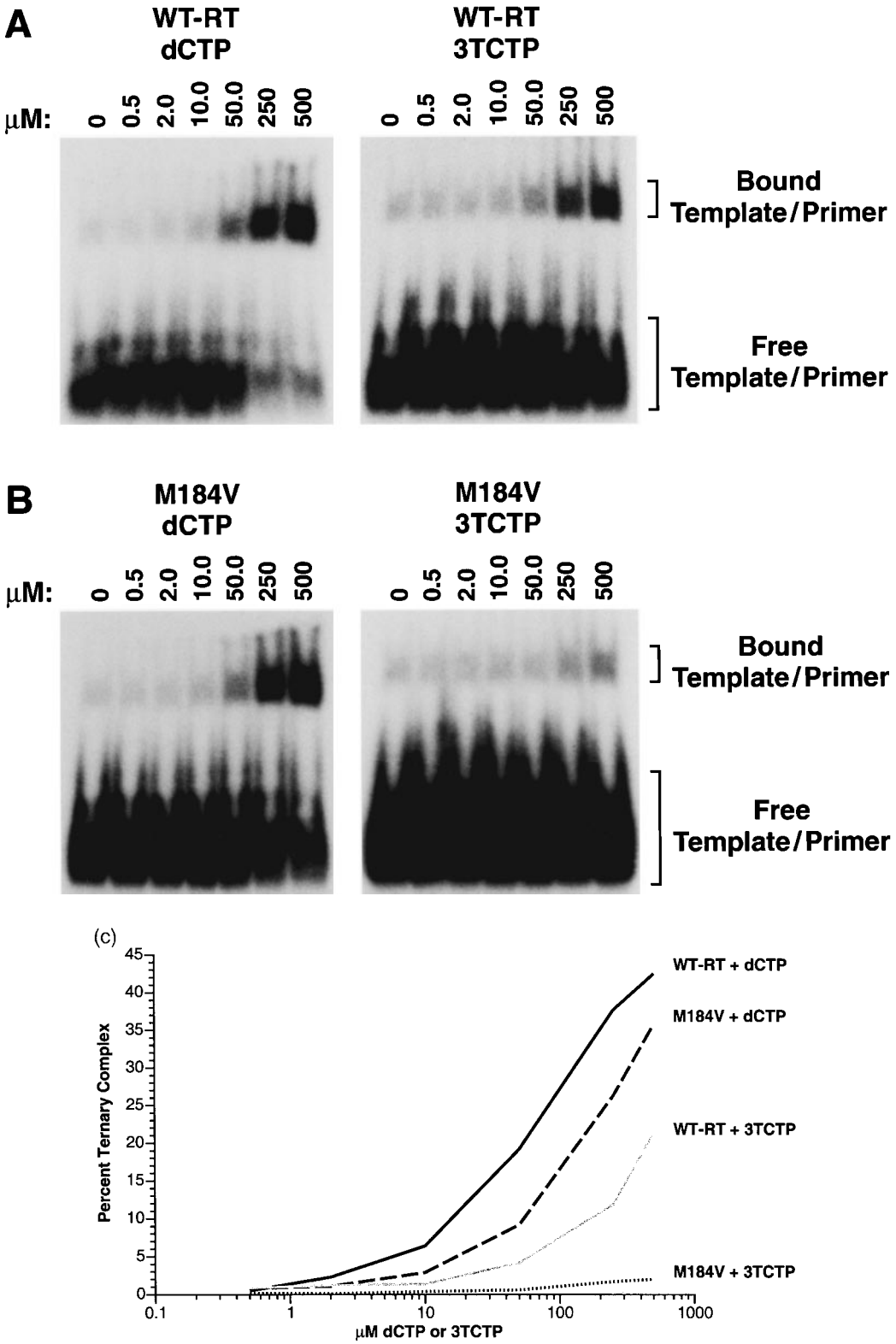

Figure 4. Gel shift assay to measure the ability of dCTP and 3TCTP to form the closed complex. HIV-1 RT (either wild-type or the M184V mutant) was allowed to form a complex with a radioactively labeled DNA substrate in which the 3′-end of the primer strand was dideoxy terminated and various concentrations of nucleoside triphosphate, either dCTP or 3TCTP (see Materials and Methods). After a five minute incubation at room temperature, the salt level was increased to 100 mM KCl and an unlabeled chase substrate, poly(rC)oligo(dG), was added. The reactions were then incubated for five minutes at 37 °C and fractioned on a 6 % Novex polyacrylamide DNA retardation gel. Autoradiographs of the gels run with reactions that contained wild-type HIV-1 RT (a) or the M184V mutant (b). (c) Graph of the data shown in (a) and (b). The radioactivity in the upper band on the gels shown in (a) and (b) was determined using a phosphorimager.

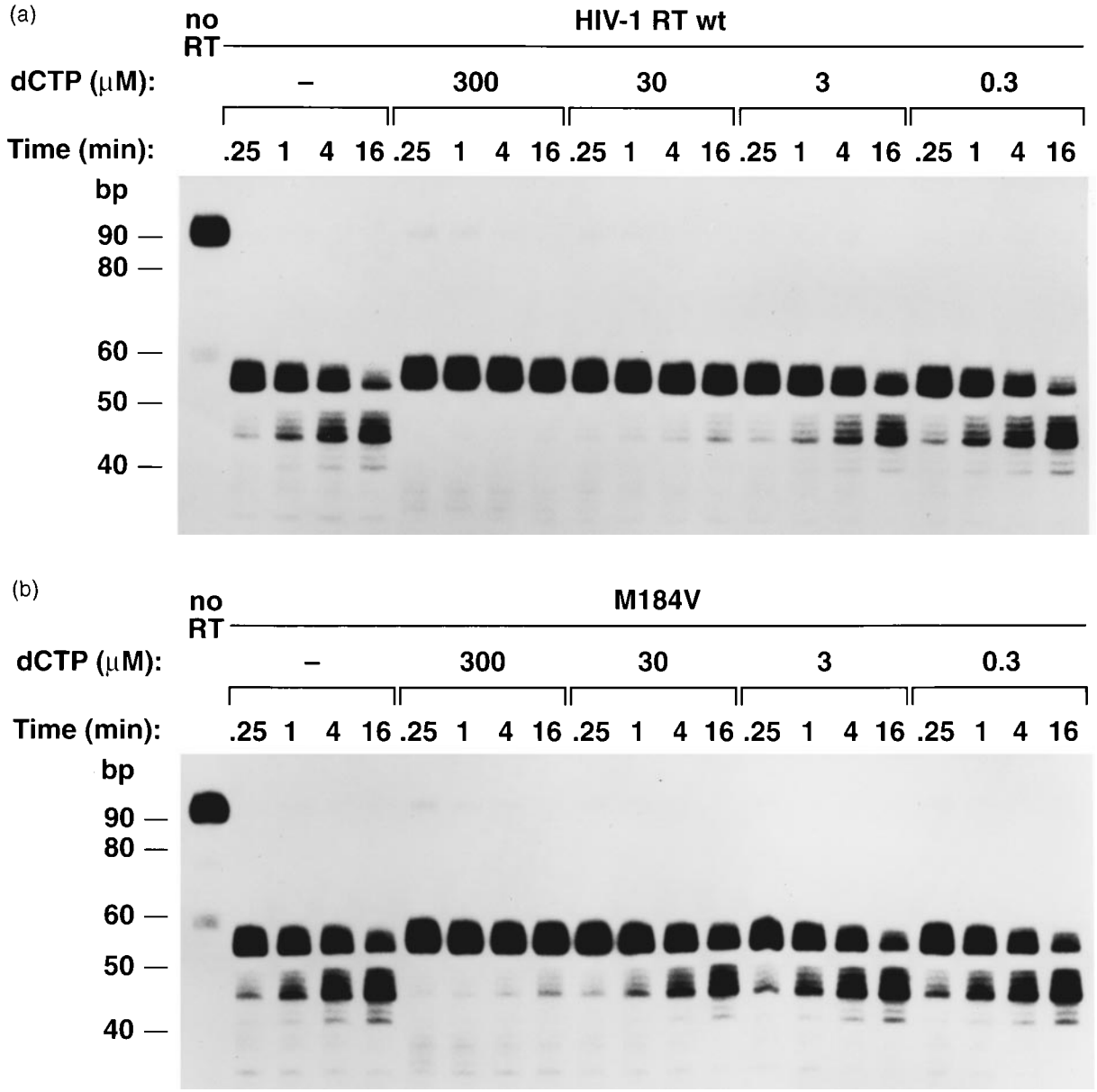

Figure 5 (*legend shown on page 414*)

β-branched amino acid residues isoleucine or valine. This leaves open the question of whether this unfavorable contact prevents the binding of 3TCTP and FTCTP or, alternatively, allows the formation of the closed complex but alters the position at which these nucleoside triphosphate analogs bind in such a fashion that catalysis is impaired.

We performed assays that were intended to measure the interactions of both wild-type HIV-1 RT and 3TC/FTC-resistant RT variants with 3TCTP. As expected, the mutant RTs showed substantial resistance to 3TCTP. We also measured the ability of the wild-type and mutant RTs to synthesize DNA at limiting dNTP concentrations. A simple interpretation of these data is that M184V and M184I mutants bind dCTP slightly less well than wild-type HIV-1 RT. Consistent with this interpretation, the RNase H and gel shift assays also show that a higher concentration of dCTP is required to induce the closed conformation with M184I and M184V mutants than with wild-type HIV-1 RT. This reduced level of affinity for normal dNTPs might be caused by some minimal steric interference between the dNTPs and the β-branched amino acid residues (isoleucine or valine) and dCTP at position 184.

In the absence of a bound dNTP, RNase H usually makes initial cleavages at positions on the template RNA strand about 17 bases from the 3′-end of the DNA primer. After the −17 cuts are made, the enzyme then makes additional cleavages

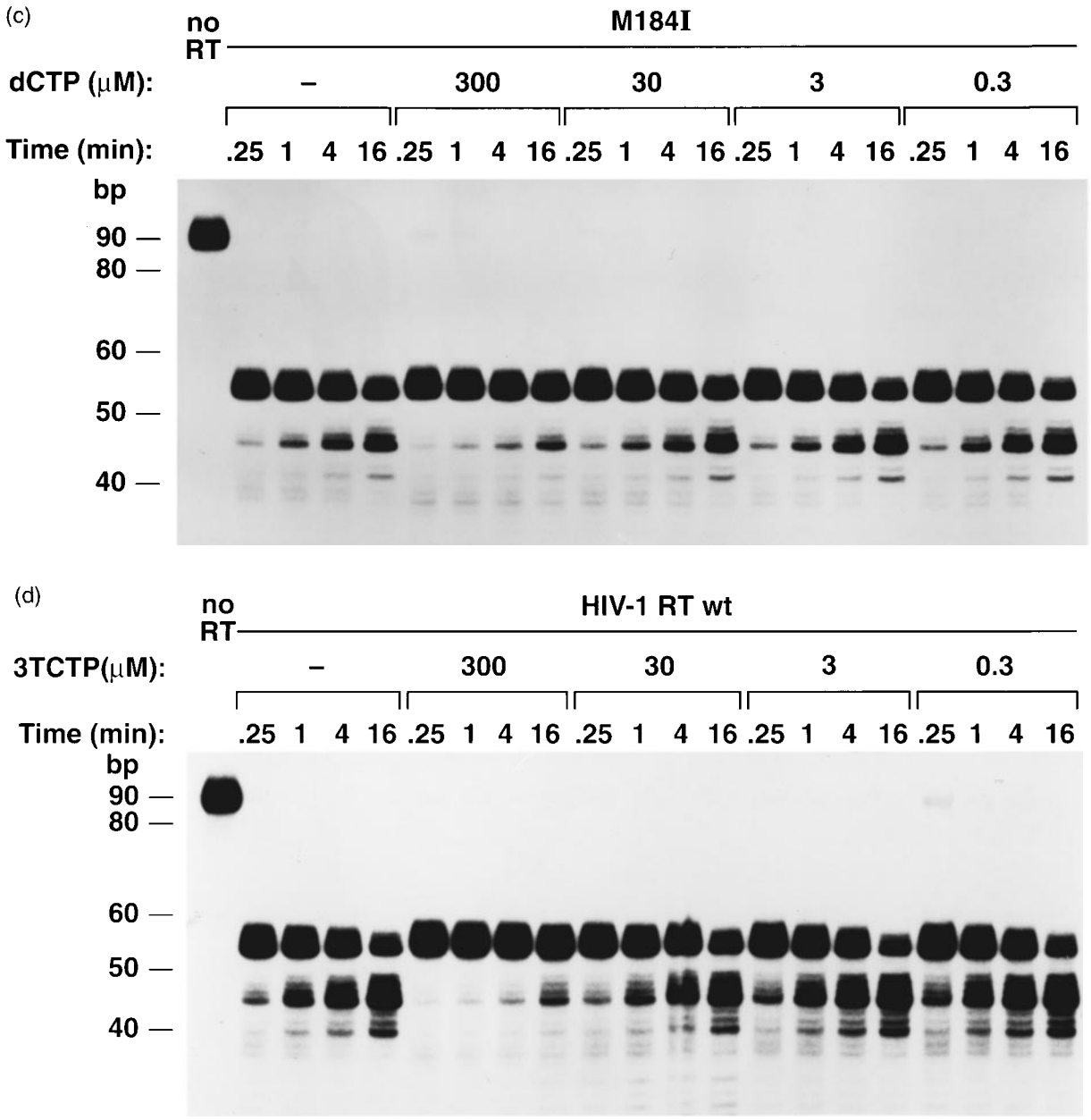

Figure 5 (*legend shown on page 414*)

approximately eight bases from the 3′-end of the primer. The −17 cleavages correspond to the position where RNase H would cleave if the 3′-end of the primer is bound at the polymerase active site (Jacobo-Molina *et al.*, 1991; Ding *et al.*, 1998; Huang *et al.*, 1998). The −8 cleavages would require some significant change in the structure of the RT/nucleic acid complex; the exact nature of this change is unclear.

If the DNA primer does not have a 3′OH group, the addition of the cognate dNTP induces the closed configuration of the enzyme, in which fingers move down onto the bound dNTP, forming the dNTP-binding pocket (Huang *et al.*, 1998). The closed configuration appears to lock the position/structure of RT on the nucleic acid, preventing the −8 cleavage. However, a comparison of the position of the −17 cleavages reveals a change in the position(s) of these cleavages: the −17 cleavages are, in the presence of a bound dNTP, moved approximately one base closer to the 3′-end of the RNA. This result is not what we expected; if there was any change, we expected that the binding of a dNTP might move the cleavage site one base closer to the 5′-end of the RNA. A simple comparison of the published RT/DNA structures, with and without bound dNTP, does not

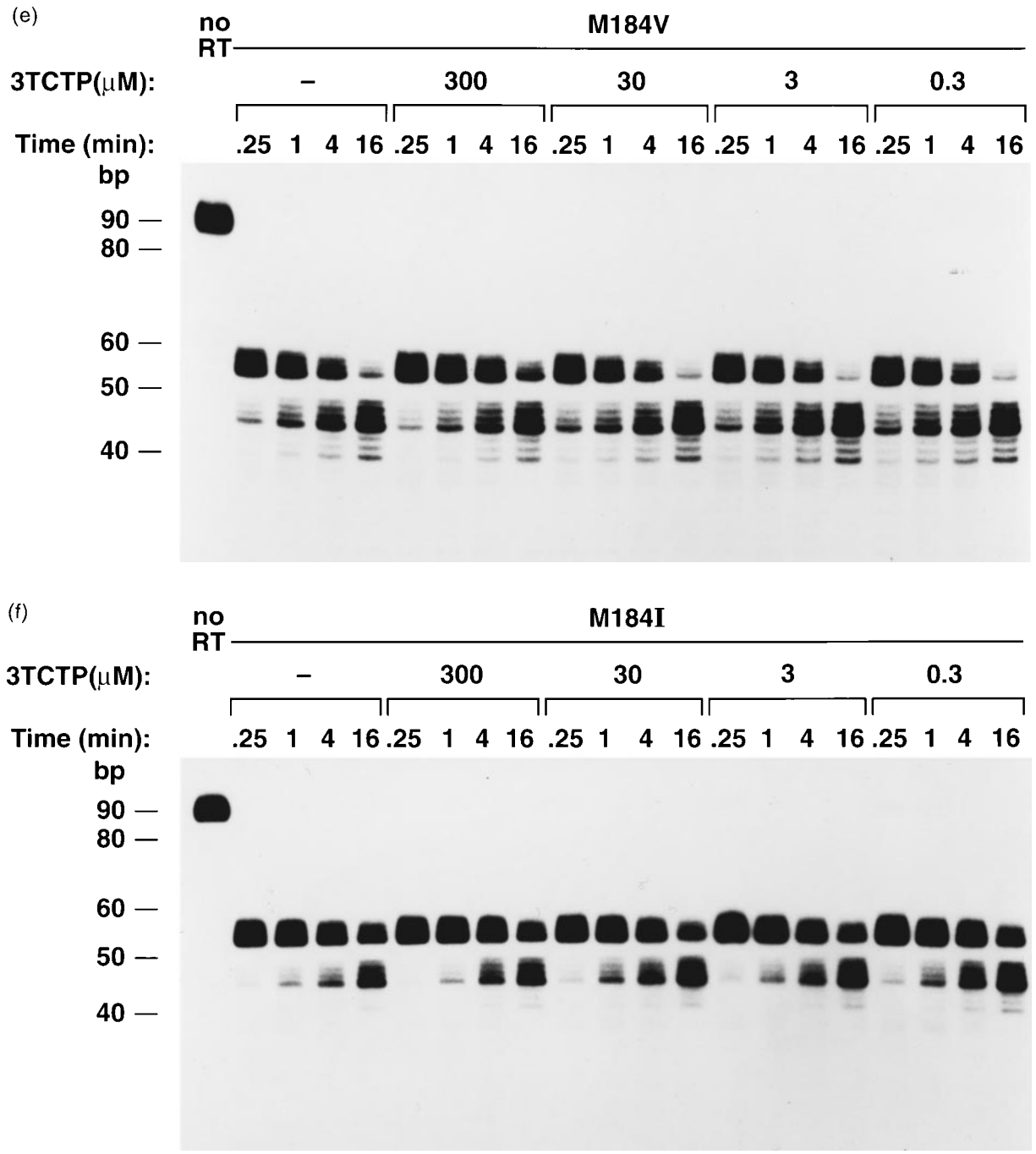

Figure 5. Effects of higher salt concentrations on RNase H cleavage in the presence of dCTP or 3TCTP. The assays were similar to those shown in Figure 3, except that the buffer was adjusted to be similar to the buffer used in the gel shift assays shown in Figure 4 (see Materials and Methods). (a)-(c) RNase H assays were done either with wild-type HIV-1 RT or the M184V or M1841 mutants, in the presence of dCTP. (d)-(f) RNase H reactions were performed with wild-type HIV-1 RT or the M184V or M184I mutants in the presence of 3TCTP.

reveal the basis for this difference in the RNA cleavage sites. However, there is an unpublished structure of HIV-1 RT in a complex with an RNA/DNA duplex and a dNTP. The structure of this complex may help us to understand the RNase H cleavages made by HIV-1 RT in the closed complex (H. Huang, G. Verdine, & S. Harris, personal communication). There is also an unpublished structure of a binary complex between HIV-1 RT and an RNA/DNA substrate (unpublished observations). However, the RNA/DNA sequences in the two structures are sufficiently different that we do not understand the one-base difference in cleavage in the binary and ternary complexes.

For both the open and the closed complex, HIV-1 RT makes three or four cleavages near −17.

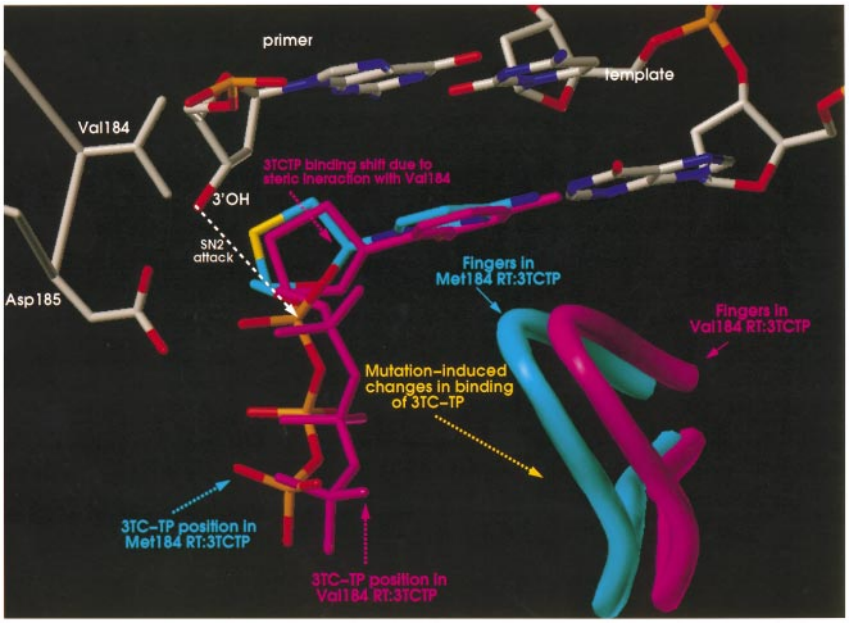

Figure 6. Model for effects of steric hindrance on 3TCTP binding by the M184V variant. The model shows one of the active-site aspartate residues (Asp185) and the adjacent residue at position 184 (valine in the Figure). The β-branched side-chain of valine causes the oxathiolane ring of 3TCTP to move from the position it would occupy when bound to the wild-type enzyme (blue) to a new position (magenta). This increases the distance to between the 3′OH of the last base of the primer and the α-phosphate group of 3TCTP. The position of the fingers (the α-carbon backbone is shown as a tube, blue for wild-type RT, magenta for the mutant) is also affected.

Although there is a one-base change in the relative position of RNase H cleavage, the closed complex cuts at several adjacent positions. If we assume that the closed complex is a stable structure in which the position of the 3′-end of the primer is fixed relative to the polymerase active site, then the ability of RNase H to cleave several positions near −17 suggests there is sufficient flexibility in HIV-1 RT (or in the nucleic acid) so that the distance between the polymerase active site and the RNase H active site can vary by several base-pairs. In this model, the multiple cleavages do not come from a repositioning or sliding of RT on the nucleic acid, but from a change in configuration of RT and/or the nucleic acid. RT makes a comparable number of distinct cleavages in the open and closed complexes (three to four). This suggests, for the open complex as well as for the closed complex, it is the changes in the conformation of the protein and/or the nucleic acid, not the movement of the protein relative to the nucleic acid, that is responsible for the multiple cleavages near −17.

Two groups who have used presteady-state kinetics to determine the behavior of M184V with 3TCTP obtained different results. Krebs *et al*. (1997) reported that, comparing wild-type HIV-1 RT and the M184V mutant, the major difference is not a change in binding but a change in the rate of catalysis. In contrast, using an RNA/DNA substrate, Feng & Anderson (1999) reported that the major difference between wild-type RT and M184V is a change in binding, not in the rate of catalysis. Feng & Anderson (1999) also reported K_d values for 3TCTP for both wild-type RT and M184V of 0.24(±0.03) μM and 5.2(±0.9) μM, respectively, whereas Krebs *et al*. (1997) reported K_d values for wild-type HIV-l RT for 3TCTP of 45(±18) μM and for the M184V mutant 66(±30) μM. Data have been reported for FTCTP, and, if we assume that FTCTP behaves similarly to 3TCTP, the data of Wilson *et al*. (1996) for FTCTP lend support to the idea that the primary effect is on the binding of the nucleoside analog.

We tested wild-type and mutant HIV-1 RT with 3TCTP using assays that measure the formation of the closed complex. The results we obtained with the RNase H assays show that it takes a higher level of concentration of 3TCTP to convert the M184V mutant to a closed ternary complex. The gel shift assay results suggest that the closed M184V ternary complex formed with 3TCTP is significantly less stable than either the equivalent complex formed by wild-type HIV-1 RT and 3TCTP or the complex formed by M184V and dCTP.

These data, taken together with the models based on HIV-1 RT crystal structure, can be used to propose a model in which 3TCTP can bind to the resistant RTs (M184V and M184I) and induce the closed configuration, but in a fashion that is strained relative to the configuration of a normal dNTP. In this strained configuration, the position of the 3TCTP is not appropriate for efficient catalysis. A model based on this idea is shown in Figure 6. The position of 3TCTP is significantly altered in the M184V complex and the position of the fingers is also altered. Because the closed complex is strained, a higher concentration of 3TCTP is

required to induce the closed conformation, and the closed complex, once formed, is inherently less stable and susceptible to disruption by salt. A comparison of the RNase H assays suggests that salt may also modestly affect the binding of 3TCTP by wild-type HIV-1 RT. The gel shift assay also shows that the wild-type enzyme binds 3TCTP less well than dCTP. Both of these results may reflect the fact that the active site of HIV-1 RT was selected for its ability to accommodate a normal dNTP, not 3TCTP.

Materials and Methods

Preparation of HIV-1 RT

The open reading frames encoding wild-type HIV-1 RT and each of the M184 mutants ere cloned into a plasmid similar to p6HRT-PROT (Le Grice & Grüninger-Leitch, 1990; Boyer *et al*., 1992, 1994). The plasmid is based on the expression vector pT5 m, and was introduced into the *Escherichia coli* strain BL21 (DE3) pLysE (Studier & Moffatt, 1986; Rosenberg *et al*., 1987; Le Grice & Grüninger-Leitch, 1990; Boyer *et al*., 1994). After induction with isopropyl β-D-thiogalactopyranoside, the plasmid expresses both the p66 form of HIV-1 RT (either wild-type or a mutant) and HIV-1 PR. Approximately 50 % of the overexpressed p66 RT is converted to the p51 form by HIV-1 PR, and p66/p51 heterodimers accumulate in *E. coli*. The p66/p51 heterodimers were purified by metal chelate chromatography (Le Grice & Grünger-Leitch, 1990; Le Grice *et al*., 1991; Boyer *et al*., 1994).

Construction of plasmid used to synthesize RNA *in vitro*

A 35-base fragment from the HIV-1 provirus clone pNL 4-3 (Adachi *et al*., 1986), including the polypurine tract from the HIV-1 genome (positions 9049-9083), was linked to 30 adenine residues and inserted into the *Eco*RI and *Hin*dIII restriction sites of plasmid pGEM-3Zf (Promega, Madison, WI). The sequence and structure of the resulting plasmid, pGPA35, were verified both by restriction enzyme mapping and by dsDNA sequencing (Gao *et al*., 1998).

RNA template preparation

The RNA template used in the RNase H cleavage assays was prepared from linearized plasmid DNA (pGPA35) by *in vitro* run-off transcription with T7 RNA polymerase, using MEGAscripts RNA synthesis kit (Ambion, Inc., Austin, TX) in the presence of 120 μCi of [α-^{32}P]UTP. The transcription reaction was heat inactivated at 70 °C for 20 minutes. The resulting RNA was purified using the PolyA Tract mRNA Isolation System composed of biotinylated oligo(dT) and streptavidin-coated magnetic particles (Promega, Madison, WI). The amount of radioactive UTP incorporation into RNA was determined by scintillation counting (Gao *et al*., 1998).

DNA oligonucleotide preparation

DNA oligonucleotides were synthesized by BioServe Biotechnologies (Laurel, MD). The lyophilized oligonucleotides were dissolved in diethylpyrocarbonate-treated water and stored at −20 °C.

RNase H cleavage assays

A sample of 50,000 cpm of the ^{32}P-labeled RNA template synthesized from linearized pGPA35 (~100 ng) was hybridized to approximately 20 ng of the individual oligonucleotide as described above in the presence of 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 2.0 mM DTT, 100 μg/ml acetylated BSA, and 10 mM Chaps. The mixtures of RNA and oligonucleotides were heated to 70 °C for ten minutes and then slowly cooled to room temperature. The reactions were initiated by adding 45 ng of purified wild-type or mutant HIV-1 RT and $MgCl_2$ to a final concentration of 5 mM in a final volume of 12 μl, and were then incubated at 37 °C. Samples were removed at 0.25, 1, 4, and 16 minutes and the reactions were terminated by adding 2 × RNA loading buffer. The products were heat denatured and separated on a denaturing 15 % polyacrylamide/7 M urea gel in Tris-borate/EDTA buffer at 1600 V for approximately 90 minutes (Gao *et al*., 1998). The gel was dried and autoradio graphed for several hours or overnight.

RNase H cleavage inhibition assay

The RNase H inhibition assay is based on a standard RNase H assay (Gao *et al*., 1998). Purified wild-type or mutant HIV-1 RT (120 ng) was first mixed with dCTP or 3TCTP at different concentrations, as indicated in the legend to Figure 1, and incubated at room temperature for two minutes. The reactions were initiated by adding annealed ^{32}P-labeled RNA template and DNA oligonucleotide in the presence of 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2.0 mM DTT, 75 μg/ml acetylated BSA, 10 mM Chaps, and 5 mM $MgCl_2$. Aliquots of the reactions were removed at 0.25, 1, 4, 16 minutes and the reactions were terminated by adding these aliquots to equal amounts of 2 × RNA loading buffer. The products were heat denatured and separated as described above (see also Gao *et al*., 1998). Salt conditions for the high-salt RNase H assay were similar to those used in the gel retardation assay. The high-salt RNase H cleavage inhibition assay was performed in 50 mM Tris-HCl, 100 mM KCl, 20 mM $MgCl_2$, 2.5 mM DTT, 75 μg/ml acetylated BSA, 10 mM Chaps, and 2.5 % (v/v) glycerol.

Polymerization assays

3TCTP inhibition

For each sample, 0.25 μg of single-stranded M13mp18 DNA (New England Biolabs, Beverly, MA) was hybridized to 0.5 μl of 1.0 OD/ml of the −47 sequencing primer (New England Biolabs) by heating to 96 °C and slowly cooling to room temperature. The template-primer was extended by adding 1.0 μg of wild-type or mutant HIV-1 RT in 25 mM Tris (pH 8.0), 75 mM KCl, 8.0 mM $MgCl_2$, 100 μg/ml BSA, 10 mM Chaps, 2.0 mM DTT, 10 μM each of dATP, dGTP, and dTTP, 2.0 μM [α-^{32}P]dCTP, and the indicated concentrations of 3TCTP (Moravek Biochemicals, Brea, CA) in a 100 μl reaction volume. The mixture was incubated at 37 °C for 30 minutes. The reaction was halted by the addition of 3 ml of ice-cold TCA and the precipitated DNA was collected by suction filtration through Whatman GF/C glass filters. The amount of incorporated radioactivity was determined by liquid scintillation counting.

Low dNTP extension assay

For each sample, 0.5 μl of 1.0 OD/ml of the −47 primer (New England Biolabs) was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After incubating at 37 °C for 30 minutes, the primer was separated from the unincorporated nucleotide by passage through Sephacryl S-200 HR (Sigma, St. Louis, MO), then 0.25 μg of single-stranded M13mp18 DNA (New England Biolabs) was annealed to the labeled primer as described above.

For each sample, 1.0 μg of wild-type RT or RT variant was added to the labeled template-primer in 25 mM Tris-HCl (pH 8.0), 75 mM KCl, 8.0 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA, and 10 mM Chaps. The reaction mixture was supplemented with 0.1, 0.5, or 2.0 μM each of dATP, dTTP, dCTP, and dGTP. The reactions were allowed to proceed at 37 °C for 15, 30, or 60 minutes, then were halted by phenol/chloroform extraction. The samples were precipitated by the addition of one volume of isopropanol, fractionated by electrophoresis on a 6% polyacrylamide and autoradiographed.

Detection of the ternary complex by gel electrophoresis

The procedure used was similar to that described by Tong *et al*. (1997). For each sample, 2.4×10^{-5} nmol of primer (5′ CTA CTA GTT TTC TCC ATC TAG ACG ATA CCA GA 3′) was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled primer was separated from unincorporated nucleotide by passage over Sepharose 22-HR (Sigma), then was annealed to a two- to threefold excess of template (5′ GAG TGC TGA GGT CTT CGC TCT GGT ATC GTC TAG ATG GAG AAA ACT AGT AG 3′) by heating and slow cooling. The underlined cytosine base in the template sequence is the first base after the 3′-end of the primer in the annealed template-primer.

The end of the primer strand was then blocked by the addition of ddGTP using 1.0 μg of wild-type RT in 1 × RT buffer (25 mM Tris (pH 8.0), 75 mM KCl, 8.0 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA and 10 mM Chaps) supplemented with 20 μM ddGTP. The reaction was incubated at 37 °C for 30 minutes, then stopped by extraction with phenol/chloroform. Excess ddGTP was removed by passage through Sephacryl S-200 -HR, followed by isopropanol precipitation and an ethanol wash.

For each sample, 10 μg of wild-type or mutant HIV-1 RT was allowed to bind to 1.5 nM of the labeled template-primer (the primer strand had a dideoxy nucleoside at the 3′-end) in 40 mM Hepes (pH 7.0), 60 mM KCl, 20 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA, 10 mM Chaps, 2.5% glycerol, and the indicated amount of dCTP or 3TCTP (0-500 μM) for five minutes at room temperature.

The shifted complex was either a binary (RT + template-primer) or ternary (RT + template-primer + nucleotide) complex. To decrease the amount of the binary complex, additional KCl (to 100 mM final) was added as well as an unlabeled chase substrate (poly(rC) oligo(dG) to 0.8 unit/ml final concentration). The mixture was incubated at 37 °C for five minutes, then 4 μl of loading buffer (Novex, San Diego, CA) was added to each sample and the samples were fractionated by electrophoresis on a 6.0% polyacrylamide DNA retardation gel (Novex). The amount of template-primer in the bound form and the amount free was determined by using a phosphorimager. The gels were then autoradiographed.

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