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Subunit-Specific Mutagenesis of the Cysteine 280 Residue of the Reverse Transcriptase of Human Immunodeficiency Virus Type 1: Effects on Sensitivity to a Specific Inhibitor of the RNase H Activity

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Treatment of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) with *N*-ethylmaleimide (NEM) selectively inhibits the RNase H activity. The cysteine residue at position 280 (C280) is the target for NEM; HIV-1 RT carrying the mutation C280S is resistant to NEM. Since HIV-1 RT is composed of two related subunits (p66 and p51) that play distinct roles, we asked whether the C280 in p51 or the C280 in p66 is responsible for the sensitivity of the enzyme to NEM. HIV-1 RT versions were prepared that had one mutant and one wild-type subunit. When these chimeric enzymes were tested, both the p51 and p66 subunits were found to contribute to the sensitivity of the enzyme to NEM. The implications of these results are discussed in the context of the structure of the enzyme.

Reverse transcriptase (RT), a key enzyme in the life cycle of human immunodeficiency virus (HIV), is responsible for the conversion of the single-stranded RNA viral genome into double-stranded DNA. The enzyme has two distinct catalytic functions: DNA polymerase and RNase H activities (7, 11, 28). Each of these activities is essential for the replication of HIV, the etiologic agent of AIDS. RT is a primary target for the chemotherapy of AIDS. The majority of the anti-RT drugs that have been developed can be divided into two classes: nucleoside analogs and nonnucleoside inhibitors (8, 12). The rapid emergence of drug resistance variants (8, 25) has emphasized the need for better drugs and thus for a better understanding of the structure and function of HIV RT.

The RT of HIV-1 is an asymmetric heterodimer consisting of a 66-kDa subunit (p66) and a 51-kDa subunit (p51). The p51 subunit is a proteolytic product of p66 (or of some larger precursor) and lacks the RNase H domain of p66 (9, 20). Although both subunits share the same N-terminal amino acid sequence, X-ray crystallographic analysis has shown that they play different structural roles in the HIV-1 RT heterodimer (1, 16, 18, 24, 27). The p51 subunit lacks both a DNA binding cleft and a functional polymerase active site (16). We have previously reported that the C280S mutant of RT did not significantly affect the catalytic properties of either polymerase or RNase H (22). However, the variant of HIV-1 RT carrying the C280S mutation is resistant to *N*-ethylmaleimide (NEM) and the natural product illimaquinone, both of which inhibit the RT-associated RNase H activity of wild-type HIV-1 RT (14, 21, 23). Analysis of the three-dimensional structure of HIV-1 RT indicates that modification of C280 in either subunit could potentially affect RNase H activity. C280 lies near an important point of contact between the RNase H domain and the thumb subdomain of the p51 subunit (14). However, C280 in the p66 subunit lies near a point of contact between the enzyme and its nucleic acid substrate. The majority of the interactions between HIV-1 RT and the nucleic acid substrate involve the polymerase domain of p66; it is possible that modification of the C280 residue in p66 could perturb the interaction of the enzyme and the nucleic acid, and this could interfere with the RNase H activity.

Since the p66 and p51 subunits play quite different roles in HIV-1 RT, it was of interest to find out which of the two subunits is involved in the sensitivity (and resistance) to compounds that interact with the C280 residue. RT heterodimers were generated that have a C280S mutation in only the p66 or the p51 subunit. They were tested to determine which subunit is involved in the resistance of the C280S mutant to NEM.

Results and discussion. We have already demonstrated that the RNase H activity of HIV-1 RT can be inactivated by NEM (13, 14, 21, 23). Both the polymerase and RNase H activities of C280S are quite similar to those of wild-type HIV-1 RT; however, the C280S mutant is resistant to NEM (14, 22). To test whether the C280S mutation confers resistance via the change in the p51 or the p66 subunit of the RT heterodimer, we used vectors that allow us to generate p66/p51 heterodimers containing one mutant subunit and one wild-type subunit. The two RT expression plasmids pUC12N/p66 (His) (with a six-histidine extension at the C terminus) and pUC12N/p51 have been described previously (3). These plasmids were used to express HIV-1 RT with either a wild-type p66 subunit [pUC12N/p66 (His)] or a wild-type p51 subunit (pUC12N/p51). A DNA segment encoding the C280S p66 subunit (with a six-histidine extension) was inserted into the polylinker region of the plasmid pUC12N/p51. The resulting plasmid expresses a mutant p66 subunit and a wild-type p51 subunit ($p66^{mut}/p51^{wt}$). Similarly, a segment encoding a C280S p51 subunit was inserted into the polylinker region of plasmid pUC12N/p66 (His) to produce $p66^{wt}/p51^{mut}$. The wild-type heterodimer was expressed by cloning a wild-type p51 coding region into vector pUC12N/p66 (His) to generate the clone p66 (His)/p51 ($p66^{wt}/p51^{wt}$). The wild-type HIV-1 RT and the variants carrying the C280S mutation in either the p66 or the p51 subunit were

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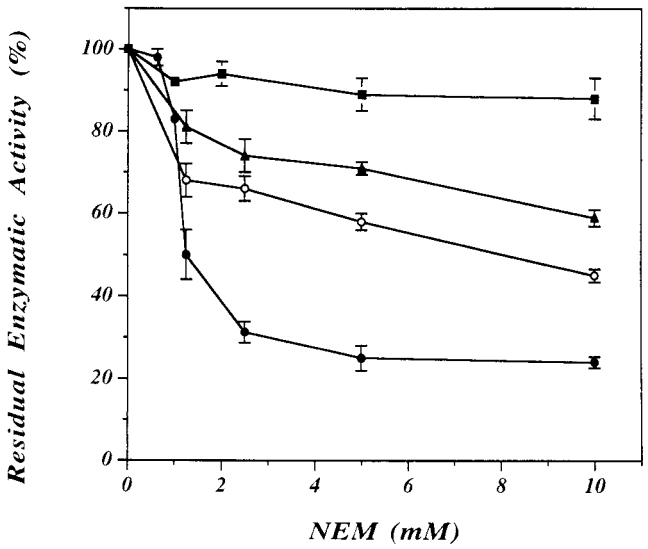

FIG. 1. Sensitivity of the C280S subunit-specific mutants of HIV-1 RT to NEM. The RNase H activity was assayed as described previously (13) by measuring the release of trichloracetic acid-soluble material from the synthetic substrate [^{3}H]poly(rA)$_n$ · poly(dT)$_n$. In all of the inhibition experiments, the enzymes were preincubated for 5 min at 30°C in the presence or absence of various concentrations of the inhibitor. The RNase H reactions were initiated by adding the substrate with dithiothreitol. Reaction mixtures were incubated at 37°C for 30 min. Enzymatic activity was calculated relative to the linear reaction rate obtained when no inhibitor was present in the reaction. One unit of RNase H activity was defined as the amount of enzyme that catalyzes the release of 1 pmol of AMP in 30 min at 37°C under the assay conditions. The graph shows the residual RNase H activity obtained with the wild-type heterodimer (p66^{wt}/p51^{wt} [●]), the heterodimer with the C280S mutation in the p51 subunit (p66^{wt}/p51^{mut} [○]), the heterodimer with the mutation in p66 (p66^{mut}/p51^{wt} [▲]), or the heterodimer with the mutation in both subunits (p66^{mut}/p51^{mut} [■]). The data represent mean values with standard deviations of at least four separate experiments for each HIV-1 RT heterodimer assayed.

purified by Ni^{+2}-nitriloacetic acid chromatography as described previously (15, 19), followed by carboxymethyl-Sepharose ion-exchange chromatography. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the final preparation of the wild-type and mutant forms of HIV-1 RT showed that the purified heterodimers were essentially homogeneous and contained equal amounts of p51 and p66 (data not shown). HIV-1 RT with the C280S substitution in both subunits is routinely used for X-ray crystallographic analyses (6); an aliquot of this material was used in the experiments described here.

We measured the effect of NEM on the RNase H activity of the wild-type enzyme (p66^{wt}/p51^{wt}), the doubly mutant enzyme (p66^{mut}/p51^{mut}), and the two mutant chimeric heterodimers, p66^{wt}/p51^{mut} and p66^{mut}/p51^{wt}, using [^{3}H]poly(rA)$_n$ · poly(dT)$_n$ as a substrate. Activity was measured by release of trichloroacetic acid-soluble radioactivity. As expected, the wild-type enzyme was sensitive, and the double mutant was resistant. As shown in Fig. 1, both of the chimeric mutants exhibit reduced sensitivity to NEM compared to the wild-type heterodimer. Resistance to NEM is enhanced when the amino acid substitution is present in the p66 subunit (p66^{mut}/p51^{wt}). This mutant enzyme retains more than 50% of its activity in 10 mM NEM, which is significantly more resistant than the wild-type heterodimer (50% inhibitory concentration [IC_{50}] of 1.3 ± 0.2 mM). The chimeric heterodimer containing the wild-type p66 and mutant p51 displayed a partial resistance to NEM; there was a sixfold increase in the IC_{50} (IC_{50} of 7.9 ± 0.4 mM) relative to the wild-type heterodimer.

Although monitoring RNase H activity in solution with a labeled homopolymeric RNA and its DNA complement is simple and convenient, additional information can be obtained with RNase H substrates in which the RNA strand is a heteropolymer of defined sequence which has been hybridized to complementary DNA. Because RT preferentially binds near the 3′ end of a primer, we have developed an assay system in which the substrate is a segment of RNA whose sequence is derived from the U3 and R regions of the HIV-1 genome. This RNA is uniformly labeled with [α-^{32}P]UTP during synthesis in vitro. The labeled RNA is then hybridized with a series of seven complementary DNA oligonucleotides, which provide multiple binding sites for RT. The cleavage products are fractionated on a sequencing gel and detected by autoradiography. This assay system provides a simple way of gauging both the activity and specificity of RNase H cleavage. A detailed description of the assay will be published elsewhere (9a).

We used this assay to measure the effects of NEM on wild-type HIV-1 RT, on the mutant with both subunits modified, and on versions of RT in which the mutations were present only in p66 or only in p51. As expected from the measurements of the RNase H activity of HIV-1 RT in solution, the wild-type enzyme was quite sensitive to NEM, and the enzyme with mutations in both subunits was quite resistant. Both of the mutants with the C280S substitution in only one subunit (p66^{wt}/p51^{mut} and p66^{mut}/p51^{wt}) were partially sensitive. This assay also showed that some of the cleavages made by the wild-type enzyme are relatively resistant to NEM (Fig. 2). This is also true of the mutant RTs in which only one subunit was modified. The pattern of fragments suggests that the cleavages that are relatively resistant to inhibition are similar for wild-type HIV-1 RT and the p66^{wt}/p51^{mut} and p66^{mut}/p51^{wt} single mutants; we suggest that these are sites in the substrate at which it is particularly easy for the RNase H of HIV-1 RT to cleave.

The level of NEM necessary to inhibit the RNase H activity was also lower in the gel assay than with the heteropolymeric template/primer. We do not believe that the results obtained in the two assays are fundamentally different. In the assay in which the release of trichloroacetic acid-soluble RNA fragments is measured, NEM was able to inhibit approximately 80% of the RNase H activity of RT (Fig. 1). However, relatively small RNA fragments can be precipitated by trichloroacetic acid. The defined template-gel assay is a much more sensitive assay for RNase H; as such, it is a more sensitive test for modest changes in the inhibition of RNase H activity.

The resistance of RNase H to inhibition by NEM appears to be associated with both the p66 subunit and the p51 subunit. In this respect, the ability of the C280S mutation to confer resistance to NEM differs from the ability of mutations that confer resistance to both nucleoside analogs and nonnucleoside inhibitors (in which the effects are conferred exclusively by changes in one of the two subunits). As predicted from the structure of HIV-1 RT, mutations that confer resistance to nucleoside analogs act through the p66 subunit (5), as do most of the mutations that confer resistance to nonnucleoside inhibitors. There is, in the case of the nonnucleoside inhibitors, one exception—the E138K mutation, which confers drug resistance only when it is present in the p51 subunit. Residue 138 is located in the portion of p51 that forms part of the nonnucleoside inhibitor binding pocket in the RT heterodimer (3, 24, 26).

The C280S mutation lies within the polymerase domain. However, chemical modification of this region affects RNase H

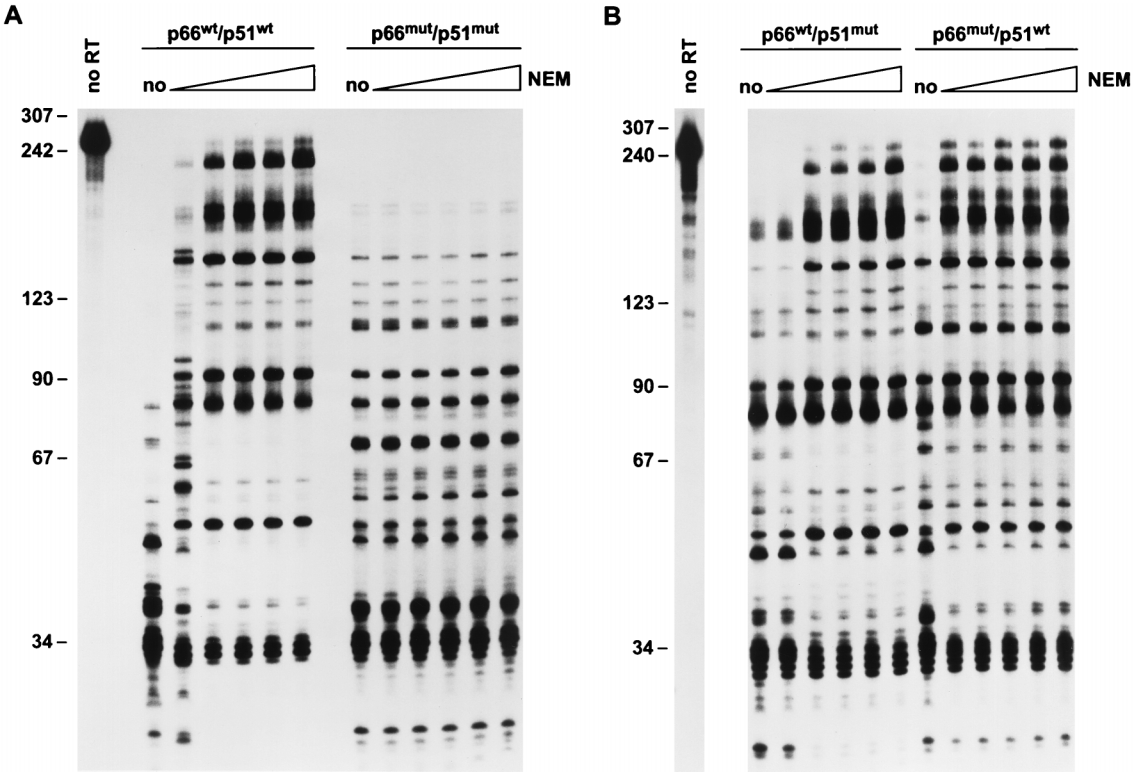

FIG. 2. Effects of NEM on the ability of wild-type and mutant HIV-1 RT to cleave a heteropolymeric substrate. In each reaction, 50,000 cpm (approximately 100 ng) of α-^{32}P-labeled RNA was annealed with 40 ng of seven oligonucleotide primers by being heated to 70°C for 10 min and then slowly cooling. The reaction was performed with 50 mM Tris-Cl, 50 mM NaCl, 2.0 mM dithiothreitol, 100 μg of acetylated bovine serum albumin, and 10 mM CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate; pH 8.0}. The RT preparations were incubated in the absence of drug (lanes labeled "no" in the figure) or in the presence of (from left to right) 50 μM, 150 μM, 1.5 mM, or 5 mM NEM for 5 min. The reactions were initiated by addition of the RT to the template-primer and incubation for 4 min at 37°C. The reactions were terminated by addition of 2× RNA loading buffer (1 ml of formamide, 1 mg of xylene cyanol, 1 mg of bromophenol blue, 100 μl of 0.5 M EDTA [pH 8.0]) and heating this mixture to 100°C for 3 min. The digestion products were fractionated on a 6% sequencing gel and detected autoradiographically. We attempted to adjust the amount of purified HIV-1 RT in the various digests to give similar levels of digestion in the absence of drug. In the reactions with wild-type HIV-1 RT, 40 ng of enzyme was used; for those with p66^{mut}/p51^{mut} RT, 40 ng of protein was used; for those with p66^{wt}/p51^{mut} RT, 60 ng was used; and for those with p66^{mut}/p51^{wt} RT, 47 ng was used.

activity without measurably affecting polymerase activity (2, 14). The X-ray crystal structures of HIV-1 RT heterodimers show that C280 lies near the middle of α helix I in the thumb subdomains of both p66 and p51. The thumb of the p51 subunit lies next to the RNase H domain of the p66 subunit. It is probably not surprising that removal of even a relatively small segment of the C terminus of RNase H affects RNase H activity (10). However, it is also true that relatively small deletions in the C terminus of the p51 subunit also affect RNase H activity (17). Based on these observations, it is relatively easy to see how changes in helix I of the p51 thumb (for example, a modification of C280 with NEM) could affect the structure of the RNase H domain (Fig. 3). Alterations in RNase H structure might distort the active site so that it can no longer cleave the RNA strand of the template/primer efficiently. It is possible that alterations in the RNase H structure could prevent the RNase H active site from making effective contact with some of the sites on the RNA strand of an RNA-DNA hybrid. It is also possible, since the template/primer is expected to pass over the p51 thumb, that chemical modification of the C280 residue in the p51 thumb may prevent the RNA strand from making proper contact with the RNase H active site. These scenarios for the effects of a modification to C280 in the thumb of p51 are not necessarily mutually exclusive.

The thumb of p66 grips the template/primer. Helices H and I of p66 contact the sugar-phosphate backbones of the template/primer with helix H contacting the primer strand and helix I contacting the template strand (16). We have proposed that mutations in the palm region of the polymerase domain, which preferentially affect RNase H activity, alter the positioning of the template/primer in a fashion that affects the interaction(s) between the template/primer and the RNase H active site (4).

We believe that a similar mechanism is responsible for the effects seen when C280 in the p66 thumb is modified by NEM. It is likely that the modification distorts helix I and alters the interaction between helix I and the template/primer. This would lead to an alteration of the position of the template/primer. The alteration must be subtle, because the repositioning does not alter the activity of the polymerase, and because, if a relatively sensitive assay is used, it is possible to detect significant residual RNase H activity. However, RNase H activity is affected. Since the thumb is closer to the polymerase active site than to the RNase H active site, this modification could have a greater effect on the position of the template/primer near the RNase H than the polymerase active site does.

The observation that both of the C280 residues, the one in the p66 subunit and the one in the p51 subunit, contribute to the sensitivity of the RNase H of HIV-1 RT to NEM underscores the importance of studying the contributions of both the p66 and p51 subunits. These data also point to the importance of the thumb. Not only is the thumb of p66 of critical impor-

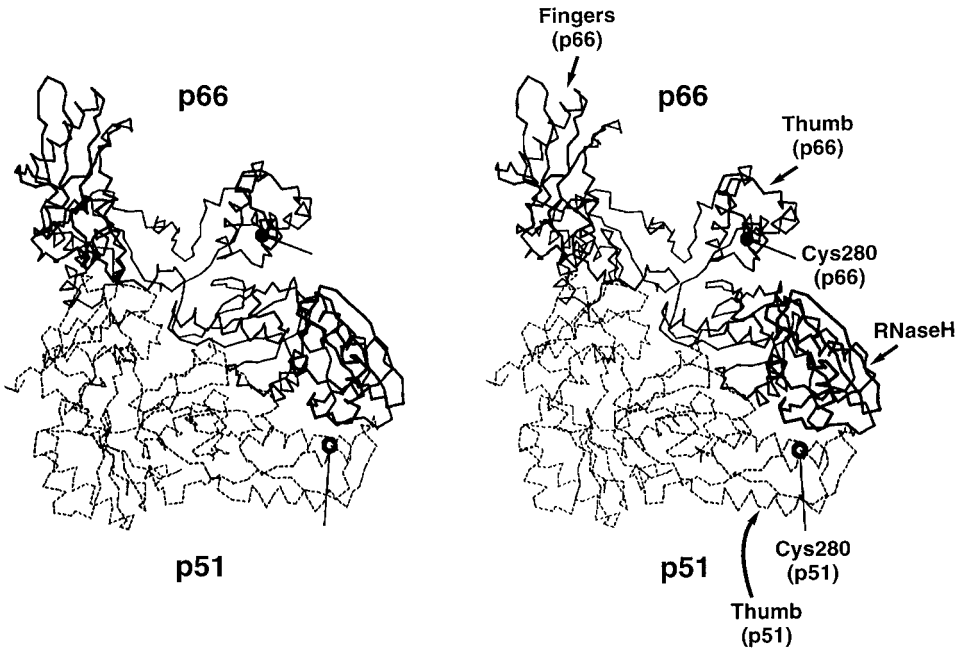

FIG. 3. Location of position 280 in the HIV-1 RT heterodimer. The stereo pair is based on the crystal structure of HIV-1 RT bound to a double-stranded DNA template/primer (16). The Cα chain of the p66 subunit is shown as a heavy trace; the Cα chain of the p51 subunit is shown as a lighter trace. The DNA is omitted from this drawing. In this orientation of HIV-1 RT, the fingers of p66 are to the upper left, and the thumb of p66 (which contains C280) is to the upper right. The thumb of p51, which contains C280 (lower right), lies just below the RNase H domain of p66 (see text). In this view, the RNase H active site is on the inner aspect of the RNase H domain.

tance in its interactions with the template/primer, but the thumb of p51 has a role(s) in directing the interactions between the RNase H domain and the template/primer.

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REFERENCES

1. **Arnold, E., A. Jacobo-Molina, R. G. Nanni, R. L. Williams, X. Lu, J. Ding, A. D. Clark, Jr., A. Zhang, A. L. Ferris, P. Clark, A. Hizi, and S. H. Hughes.** 1992. Structure of HIV-1 reverse transcriptase/DNA complex at 7 Å resolution showing active site locations. Nature **357:**85–89.
2. **Barber, A. M., A. Hizi, J. V. Maizel, and S. H. Hughes.** 1990. HIV-1 reverse transcriptase: structure predictions of the polymerase domain. AIDS Res. Hum. Retroviruses **6:**1061–1072.
3. **Boyer, P. L., J. Ding, E. Arnold, and S. H. Hughes.** 1994. Subunit specificity of mutations that confer resistance to nonnucleoside inhibitors in human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **38:**1909–1914.
4. **Boyer, P. L., C. Tantillo, A. L. Ferris, E. Arnold, and S. H. Hughes.** 1994. Mutational analysis of the fingers and palm subdomains of human immunodeficiency virus type-1 (HIV-1) reverse transcriptase. J. Mol. Biol. **243:**472–483.
5. **Boyer, P. L., and S. H. Hughes.** 1996. Nucleoside-analogue resistance involves the p66 subunit of HIV-1 RT. Nature Struct. Biol. **3:**579–580.
6. **Clark, A. D., Jr., A. Jacobo-Molina, P. Clark, S. H. Hughes, and E. Arnold.** 1995. Crystallization of human immunodeficiency virus type 1 reverse transcriptase with and without nucleic acid substrates, inhibitors, and an antibody Fab fragment. Methods Enzymol. **262:**171–185.
7. **Coffin, J. M.** 1990. Retroviridae and their replication, p. 1437–1500. *In* B. N. Fields and D. M. Knipe (ed.), Fields' virology, 2nd ed. Raven Press, New York, N.Y.
8. **De Clercq, E.** 1994. HIV resistance to reverse transcriptase inhibitors. Biochem. Pharmacol. **47:**155–169.
9. **diMarzo Veronese, F., T. D. Copeland, A. L. DeVico, R. Rhaman, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1986. Characterization of highly immunogenic p66/p51 as the reverse transcriptase of HTLV-III/LAV. Science **231:**1289–1291.

9a. **Gao, H.-Q., and S. H. Hughes.** Unpublished observations.

10. **Ghosh, M., K. J. Howard, C. E. Cameron, S. J. Benkovic, S. H. Hughes, and S. F. J. Le Grice.** 1995. Truncating α-helix E′ of p66 human immunodeficiency virus reverse transcriptase modulates RNase H function and impairs DNA strand transfer. J. Biol. Chem. **270:**7068–7076.
11. **Goff, S. P.** 1990. Retroviral reverse transcriptase: synthesis, structure and function. J. Acquired Immune Defic. Syndr. **3:**817–831.
12. **Grob, P. M., J. C. Wu, K. A. Cohen, R. H. Ingraham, C. K. Shih, K. D. Hargrave, T. L. McTague, and V. J. Merluzzi.** 1992. Nonnucleoside inhibitors of HIV-1 reverse transcriptase:nevirapine as a prototype drug. AIDS Res. Hum. Retroviruses **8:**145–152.
13. **Hizi, A., R. Tal, M. Shaharabany, and S. Loya.** 1991. Catalytic properties of the reverse transcriptase of human immunodeficiency virus type 1 and type 2. J. Biol. Chem. **286:**6230–6239.
14. **Hizi, A., M. Shaharabany, R. Tal, and S. H. Hughes.** 1992. The effects of cysteine mutations on the reverse transcriptases of human immunodeficiency virus types 1 and 2. J. Biol. Chem. **267:**1293–1297.
15. **Hochuli, E., W. Bannawarth, H. Dobeli, R. Gentz, and D. Struber.** 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. BioTechnology **6:**1321–1325.
16. **Jacobo-Molina, A., J. Ding, R. G. Nanni, A. D. Clark, Jr., X. Lu, C. Tantillo, R. L. Williams, G. Kamer, A. L. Ferris, P. Clark, A. Hizi, S. H. Hughes, and E. Arnold.** 1993. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. Proc. Natl. Acad. Sci. USA **90:**6320–6324.
17. **Jacques, P. S., B. M. Wöhrl, K. J. Howard, and S. F. Le Grice.** 1994. Modulation of HIV-1 reverse transcriptase function in "selectively deleted" p66/p51 heterodimers. J. Biol. Chem. **269:**1388–1393.
18. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz.** 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256:**1783–1790.
19. **Le Grice, S. F. J., and F. Grüninger Leitch.** 1990. Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. Eur. J. Biochem. **187:**307–314.
20. **Lightfoote, M. M., J. E. Coligan, T. M. Folks, A. S. Fauci, M. A. Martin, and S. Venkatesan.** 1986. Structural characterization of reverse transcriptase and endonuclease polypeptides of the acquired immunodeficiency syndrome virus. J. Virol. **60:**771–775.
21. **Loya, S., R. Tal, Y. Kashman, and A. Hizi.** 1990. Illimaquinone, a selective inhibitor of the RNase H activity of human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **34:**2009–2012.
22. **Loya, S., R. Tal, S. H. Hughes, and A. Hizi.** 1992. The effects of cysteine

mutations on the catalytic activities of the reverse transcriptases of HIV-1. J. Biol. Chem. **267:**13879–13883.

23. **Loya, S., and A. Hizi.** 1993. The interaction of illimaquinone, a selective inhibitor of the RNase H activity, with the reverse transcriptases of human immunodeficiency and murine leukemia retroviruses. J. Biol. Chem. **268:** 9323–9328.
24. **Nanni, R. G., J. Ding, A. Jacobo-Molina, S. H. Hughes, and E. Arnold.** 1993. Review of HIV-1 reverse transcriptase three-dimensional structure: implications for drug design. Perspect. Drug Discov. Des. **1:**129–150.
25. **Richman, D. D.** 1993. Resistance of clinical isolates of human immunodeficiency virus to antiretroviral agents. Antimicrob. Agents Chemother. **37:** 1207–1213.
26. **Tantillo, C., J. Ding, A. Jacobo-Molina, R. G. Nanni, P. L. Boyer, S. H. Hughes, R. Pauwels, K. Andries, P. A. J. Janssen, and E. Arnold.** 1994. Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase: implications for mechanisms of drug inhibition and resistance. J. Mol. Biol. **243:**369–387.
27. **Wang, J., S. J. Smerdon, J. Jäger, L. A. Kohlstaedt, P. A. Rice, J. M. Friedman, and T. A. Steitz.** 1994. Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer. Proc. Natl. Acad. Sci. USA **91:**7242–7246.
28. **Whitcomb, J. M., and S. H. Hughes.** 1992. Retroviral reverse transcription and integration: progress and problems. Annu. Rev. Cell Biol. **8:**275–306.