NOTES

Reversion of a Moloney Murine Leukemia Virus RNase H Mutant at a Second Site Restores Enzyme Function and Infectivity

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Received 2 March 1995/Accepted 2 May 1995

The reverse transcriptase of retroviruses contains an RNase H activity essential for the proper synthesis of the viral DNA copy of the RNA genome. We have previously characterized a number of point mutations altering the RNase domain of the Moloney murine leukemia virus reverse transcriptase (S. W. Blain and S. P. Goff, *J. Biol. Chem.* 268:23585–23592, 1993). One such mutation, Y586F (a Y-to-F change at position 586), reduced RNase H activity, as assayed by in situ gel analysis, to about 5% of the wild-type level and prevented viral replication. We have now recovered a revertant virus with near-normal infectivity and in vitro enzymatic activity. The revertant contains a single substitution, N613H, distant in the primary sequence of the protein, but modeling with the *Escherichia coli* RNase H structure suggests that the reverted residue is close in space to the original substituted residue. Examination of the structure permits some suggestions as to how this second-site revertant restores enzyme activity.

Retroviral reverse transcriptase (RT) is the enzyme responsible for converting the single-stranded RNA genome into the double-stranded DNA form (1a, 32). In the course of reverse transcription RT utilizes both a DNA polymerase activity, which synthesizes DNA from either RNA or DNA templates, and an RNase H activity that degrades genomic RNA in RNA-DNA hybrid form (6, 28). The RNase H activity of Moloney murine leukemia virus (M-MuLV) RT is located in the C-terminal one-third of the enzyme (29). This domain has high sequence similarity with other RNases H, including those of *Escherichia coli* (21, 35) and human immunodeficiency virus type 1 (HIV-1) (9, 17, 22), and it is likely that the structure of this RNase H is very similar to those of the other RNases H (25). These structures consist of a compact globular shape with five alpha helices and five beta sheets forming two groove-like depressions. Several invariant residues implicated in the catalytic activity of the enzyme are thought to define the active sites of all RNases H. The structures were determined in the absence of substrate, but modeling with one enzyme and an RNA-DNA decamer has pinpointed residues potentially involved in substrate binding (35). In a number of studies, many of these residues have been altered by mutagenesis, and reductions in binding and/or catalysis were observed as predicted, generally supporting the structural model (3–5, 10, 18–20, 24, 26, 33).

RNases H cleave RNA to produce oligonucleotides with 5'-PO₄ and 3'-OH groups and show a strong divalent cation requirement for catalysis (8, 16, 34). The traditional substrate

for this enzyme is RNA-DNA, although retroviral RNases H have been shown to also cleave RNA-RNA duplexes (2, 3, 14) via an activity dubbed RNase H* (15). It has been suggested that the DNA polymerase domain present in retroviral enzymes is required for this RNA-RNA hydrolysis activity (3). While there is probably a single site for cleavage of RNA-DNA and RNA-RNA, differences in substrate binding have been observed; mutations which preferentially destroy RNA-DNA and not RNA-RNA nuclease activity, and vice versa, have been identified (3). We have previously reported the characterization of one such M-MuLV RT-associated RNase H mutant, Y586F (containing a conservative change of the Y residue at position 586 to the F residue found in avian RTs). In vitro, as analyzed by in situ gel techniques, this mutant has fully wild-type DNA polymerase specific activity and displayed altered RNase H substrate specificities: it was fully active on RNA-RNA substrates but retained only about 5% activity on RNA-DNA substrates (3). This mutant thus defines a region important for the discrimination between RNA and DNA on the nonhydrolyzed strand and a potential site of contact between the enzyme and the substrate. One approach to identifying additional residues that may be involved in substrate recognition and binding is to isolate second-site suppressor mutations that compensate for the original mutation. The Y586F mutant virus was noninfectious in vivo (4), and suppressor mutations could in principle be recovered in revertant viruses with improved infectivity. In this report, we described the isolation and characterization of a second-site revertant of the Y586F mutant, which now replicates normally in NIH 3T3 cells. The second-site suppressor mutation identified in this revertant restored normal enzyme activity on RNA-DNA substrates. The predicted position in space of the altered amino acid is consistent with previous substrate modeling of the M-MuLV enzyme based on the known structure of *E. coli* RNase H.

To recover a revertant virus, a cell line stably expressing the

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Y586F mutant was passaged repeatedly, and culture medium was collected and tested for the presence of replication-competent virus after infection of fresh NIH 3T3 cells (30) by measuring RT activity of the culture medium (12). Whereas the line initially released only defective virus, infectious virus spontaneously appeared in the supernatant culture media after 8 to 12 weeks. We will refer to this line as Y586F^R. Virus was harvested from the Y586F^R cell line and passaged three times in NIH 3T3 cells, to reduce the amount of noninfectious, parental virus which might have been propagated by the revertant acting as a helper. During repeated transfers, the cultures became RT positive at increasingly early time points, suggesting that the reverted virus was increasing as a percentage of the total virus pool.

Viral DNA was examined to identify the mutations responsible for the reversion event. We harvested low-molecular-weight DNA 24 h postinfection (13) and used PCR to amplify the entire RNase H domain (nucleotides 4482 through 5138) in the preintegrative viral DNA (primers 5'EcoRV [5'CCCGGC TACGCTGCTCCC3'] and 3'Int [5'GGCCCCCAACTTGGT TAGG3']; 25 cycles with an annealing temperature of 57°C). A hallmark of the original Y586F mutation, a *Bst*UI site at position 4801 in the *pol* coding region, was still present, suggesting that the Y586F^R virus had acquired another mutation at a secondary site. We subcloned the PCR-amplified DNA fragment from the *Eco*RV site at nucleotide 4535 to the *Xmn*I site at nucleotide 5087 into the pBluescript vector (Stratagene, La Jolla, Calif.) and determined the DNA sequence of the RNase H domain. The Y586F mutation and *Bst*UI site at nucleotide 4801 were indeed still present. In addition, we found a single A-to-C base change at position 4883, resulting in a predicted single amino acid change at residue 613, changing an Asn to a His (codon AAT to CAT). This mutation, termed N613H, was a candidate suppressor mutation.

To test whether the N613H mutation was responsible for the reverted phenotype of the virus, we subcloned this region from the cloned fragment of the revertant viral DNA back into pNCA, a plasmid containing a cloned copy of wild-type M-MuLV proviral DNA (7). We also generated the N613H mutation de novo by site-directed mutagenesis of the wild-type DNA, with and without the Y586F mutation. Mutant N613H was constructed by two-step PCR on a wild-type template, by amplification first with primers 5'GATCTCGTCTTTATGTT TAATCTCTTTGCCTTC3' and 5'EcoRV and then with primers 3'Int and 5'EcoRV, creating a new *Mse*I site. Y586F was similarly created with the mutant oligonucleotide 5'GCAG TAGCAAACGCGAAACGGCTATCAG3', creating a new *Bst*UI site. The presence of the mutations was confirmed by restriction mapping and sequencing. These various mutant DNAs were introduced into NIH 3T3 cells by DEAE-dextran transfection (23), and the ability of the mutant viruses to replicate was assessed by testing for the presence of RT activity in the culture supernatant (12) (Fig. 1). Wild-type DNA induced the release of progeny virus by 3 to 8 days postinfection (Fig. 1, lane 6), and the parental Y586F DNA did not produce any replication-competent virus, even after 22 days of passage (lane 4). The virus containing the Y586F^R fragment was RT positive by 8 to 14 days postinfection (lane 3). The same phenotype was seen with the recombinant double mutant N613H/Y586F, confirming that N613H was indeed a second-site suppressor of the Y586F^R mutation (lane 2). Thus, the presence of the second-site mutation restored viral infectivity to mutant Y586F, although the double-mutant virus was slightly delayed compared with the wild type. The single N613H mutant was as infectious as the wild type (lane 1), indicating that the suppressor mutation itself was not deleterious to the virus.

FIG. 1. Test of viral DNA infectivity. Proviral DNAs were assayed for infectivity by DEAE-dextran transient transfection in NIH 3T3 cells. Mock, mock transfection; WT, wild-type (pNCA) DNA. Culture supernatant was harvested at 3, 8, 14, and 22 days posttransfection as indicated on the right and analyzed for RT activity as a measure of viral spread.

The effects of the mutations on the enzymatic activities of RT were also assessed. The mutations were transferred into plasmid pRT30-2, which expresses the full-length M-MuLV RT in *E. coli* (31). Bacterial lysates were prepared, and the extracts were analyzed for RNase H activity on both RNA-DNA and RNA-RNA templates by in situ gel techniques and for DNA polymerase specific activity on oligo(dT) poly(rA) primer templates (Fig. 2). DNA polymerase specific activities of all the mutants were normal. After normalization for DNA polymerase activity, proteins were displayed on a sodium dodecyl sulfate-polyacrylamide gel cast with ³²P-labeled heteropolymeric RNA in RNA-DNA hybrid or RNA-RNA duplex form (3). After electrophoresis of the proteins, the gel was incubated in several changes of buffer to permit renaturation of the proteins and digestion of the substrate. The gel was washed extensively, dried, and exposed to autoradiography. Enzyme activity was detected as zones of clearing in the gel and quantitated by comparison with standards; levels could be safely estimated to lie within a twofold range. Y586F was severely reduced for activity on RNA-DNA substrates but exhibited full activity on RNA-RNA substrates (Fig. 2A, lanes 7 to 9), as seen previously. This result suggested that this mutation affected substrate binding in a selective way as opposed to overall catalytic activity (3). The double mutant, N613H/Y586F, showed the activity on RNA-DNA substrates at about 30% of the wild-type level and was also fully active on RNA-RNA substrates (Fig. 2A, lanes 4 to 6). The single N613H mutant was also fully active on both substrates (Fig. 2B, lanes 7 to 9, and data not shown). These results suggest that the presence of the second-site mutation restored enzyme activity both in vitro and in vivo.

We examined the three-dimensional structure of *E. coli* RNase H to determine where the homologous residues were located (35). Y-586 is conserved in most RNases H, including those of *E. coli* (residue 73) and HIV-1 (residue 501). On the

FIG. 2. Analysis of mutants in the RNA-DNA and RNA-RNA in situ gel assay. Mutants were expressed in *E. coli* in the pRT30-2 RT expression plasmid and analyzed in the in situ gel assay. WT, wild-type; DH5 α , DH5 α extract alone. (A) (Left) RNA-DNA substrate; (right) RNA-RNA substrate. The amount of enzyme loaded in a particular lane is the same for the RNA-DNA and RNA-RNA panels. (B) RNA-DNA substrate. In each panel, lanes 1, 4, and 7; lanes 2, 5, and 8; and lanes 3, 6, and 9 may be compared and correspond to decreasing twofold dilutions of wild type or mutant.

basis of the model of *E. coli* RNase H with an RNA-DNA decamer, it has been suggested that the hydroxyl group of the Y-586 residue interacts with the DNA strand of the hybrid (Fig. 3) (35). The homologous residue in the HIV-1 RT structure, Y-501, actually contacts the substrate in the three-dimensional model of HIV-1 RT complexed with the double-stranded DNA (1, 17). Without the -OH group of this tyrosine, the interaction with the DNA strand of the hybrid might not be made. This would interfere with the overall binding of the RNA-DNA substrate and account for the observed reduction in RNA-DNA nuclease activity. The -OH group from the RNA strand in the RNA-RNA duplex might permit a compensatory interaction to be made, consistent with the wild-type RNA-RNA nuclease activity seen with this mutant enzyme.

Residue N-613 of M-MuLV RT is homologous to N-100 in the *E. coli* enzyme and is 27 amino acids away from residue Y-586 in the M-MuLV primary sequence. Remarkably, modeling with the *E. coli* RNase H structure suggests that N-100 is very close in space to Y-73 and to the substrate binding cleft (Fig. 3). This observation suggests that the histidine residue of the N613H revertant makes a compensatory interaction with the substrate further along the DNA chain, effectively replacing the altered Y-586 residue. In the *E. coli* RNase H crystal, a sulfate ion is located at the N terminus of alpha helices A and D, in a site 13.7 Å (1.37 nm) away from the center of the active site. This site is thought to mark the position of a nucleotide phosphate of the DNA bound to the enzyme (35). This position is very near residues Y-73 and N-100. While it is unclear why histidine might be able to make compensatory interactions better than asparagine, it is possible that the histidine's larger size is important for making contact with the substrate. It should be noted that Y-73 has been mutated to a leucine in *E. coli* RNase H without any detectable effect on activity (20). As leucine is much smaller than either tyrosine or phenylalanine, perhaps compensatory interactions are made via an additional water molecule.

Detailed structural information about the substrate and its

contacts with enzyme is not yet available. In fact, the exact conformation of the substrate is uncertain; it has been suggested that bound substrate is profoundly bent (17) and that an RNA-DNA hybrid may adopt a novel mixed structure (H form) (11, 27). Genetic studies such as this one may provide useful information in the absence of substrate-enzyme structure. In particular, the characterization of a revertant virus has suggested a functional interaction between two residues of the

FIG. 3. Model of *E. coli* RNase H with the location of the M-MuLV point mutants. Model of the *E. coli* RNase H backbone (purple) based on data from reference 35. The side chains of the mutant and revertant residues are highlighted to depict their locations in the context of the *E. coli* RNase H enzyme. In all cases, the highlighted *E. coli* residue is the same as the residue found in wild-type M-MuLV RT at that position. D-524 = D-10 (white); Y-586 = Y-73 (turquoise); and N-613 = N-100 (yellow). An RNA-DNA decamer has been positioned in the putative binding site of the enzyme, also from reference 35. The DNA strand is in blue, and the RNA strand is in red.

enzyme. The fact that we were able to recover a revertant altered at a second residue very near in space to the original alteration suggests that this is an important area of interaction with the DNA strand of the substrate and possibly with the sugar moiety of this strand. Our findings also support the enzyme-substrate modeling performed with *E. coli* RNase H upon which the Y586F mutant was originally based and suggest that the mechanism for substrate binding and catalysis is conserved for the different RNases H.

We thank C. Bingham for helpful discussion.

This work was supported by Public Health Service grant CA 30488 from the National Cancer Institute and the Howard Hughes Medical Institute. Support for S.W.B. was provided by a PHS predoctoral training grant from the NIGMS. S.P.G. is an investigator of the Howard Hughes Medical Institute.

REFERENCES

1. Arnold, E. Personal communication.
- 1a. Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumor viruses. *Nature (London)* **226**:1209–1211.
2. Ben-Artzi, H., E. Zeelon, M. Gorecki, and A. Panet. 1992. Double-stranded RNA-dependent RNase activity associated with human immunodeficiency virus type 1 reverse transcriptase. *Proc. Natl. Acad. Sci. USA* **89**:927–931.
3. Blain, S. W., and S. P. Goff. 1993. Nuclease activities of Moloney murine leukemia virus reverse transcriptase: mutants with altered substrate specificities. *J. Biol. Chem.* **268**:23585–23592.
4. Blain, S. W., and S. P. Goff. 1995. Effects on DNA synthesis and translocation caused by mutations in the RNase H domain of Moloney murine leukemia virus reverse transcriptase. *J. Virol.* **69**:4440–4452.
5. Boyer, P. L., A. L. Ferris, and S. H. Hughes. 1992. Mutational analysis of the fingers domain of human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.* **66**:7533–7537.
6. Champoux, J. 1993. Role of ribonuclease H in reverse transcription, p. 103–116. In A.-M. Skalka and S. P. Goff (ed.), *Reverse transcriptase*. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
7. Colicelli, J., and S. P. Goff. 1988. Sequence and spacing requirements of a retrovirus integration site. *J. Mol. Biol.* **199**:47–59.
8. Crouch, R. J. 1990. Ribonuclease H: from discovery to 3D structure. *New Biol.* **2**:771–777.
9. Davies, J. F., II, Z. Hostomska, Z. Hostomsky, S. R. Jorden, and D. A. Matthews. 1991. Crystal structure of the ribonuclease H domain of the HIV-1 reverse transcriptase. *Science* **252**:88–95.
10. Dudding, L. R., and V. Mizrahi. 1993. Rapid kinetic analysis of a point mutant of HIV-1 reverse transcriptase lacking ribonuclease H activity. *Biochemistry* **32**:6116–6120.
11. Fedoroff, O. Y., M. Salazar, and B. R. Reid. 1993. Structure of a DNA:RNA hybrid duplex: why RNase H does not cleave pure RNA. *J. Mol. Biol.* **233**:509–523.
12. Goff, S. P., P. Traktman, and D. Baltimore. 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase. *J. Virol.* **38**:239–248.
13. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **106**:365–369.
14. Hostomsky, Z., G. O. Hudson, S. Rahmati, and Z. Hostomska. 1992. RNase D, a reported new activity associated with HIV-1 reverse transcriptase, displays the same cleavage specificity as *Escherichia coli* RNase III. *Nucleic Acids Res.* **20**:5819–5824.
15. Hostomsky, Z., S. H. Hughes, S. P. Goff, and S. F. J. Le Grice. 1994. Redesignation of the RNase D activity associated with retroviral reverse transcriptase as RNase H^{*}. *J. Virol.* **68**:1970–1971.
16. Jacobo-Molina, A., and E. Arnold. 1991. HIV reverse transcriptase structure-function relationships. *Biochemistry* **30**:6354–6361.
17. Jacobo-Molina, A., J. Ding, R. G. Nanni, A. D. J. Clark, 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.
18. Kanaya, S., C. Katsuda, S. Kimura, T. Nakai, E. Kitakuni, H. Nakamura, K. Katayanagi, K. Morikawa, and M. Ikehara. 1991. Stabilization of *Escherichia coli* ribonuclease H by introduction of an artificial disulfide bond. *J. Biol. Chem.* **266**:6038–6044.
19. Kanaya, S., C. Katsuda-Nakai, and M. Ikehara. 1991. Importance of the positive charge cluster in *Escherichia coli* ribonuclease H1 for the effective binding of substrate. *J. Biol. Chem.* **266**:11621–11627.
20. Kanaya, S., A. Kohara, Y. Miura, A. Sekiguchi, S. Iwai, H. Inoue, E. Ohtsuka, and M. Ikehara. 1990. Identification of the amino acid residues involved in an active site of *Escherichia coli* ribonuclease H by site-directed mutagenesis. *J. Biol. Chem.* **265**:4615–4621.
21. Katayanagi, K., M. Miyagawa, M. Matsushima, M. Ishikawa, S. Kanaya, M. Ikehara, T. Matsuzaki, and K. Morikawa. 1990. Three-dimensional structure of ribonuclease H from *E. coli*. *Nature (London)* **347**:306–309.
22. 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.
23. McCutchan, J. H., and J. S. Pagano. 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran. *J. Natl. Cancer Inst.* **41**:351–357.
24. Mizrahi, V., R. L. Brooksbank, and N. C. Nkabinde. 1994. Mutagenesis of the conserved aspartic acid 443, glutamic acid 478, asparagine 494, and aspartic acid 498 residues in the ribonuclease H domain of the p66/p51 human immunodeficiency virus type 1 reverse transcriptase: expression and biochemical analysis. *J. Biol. Chem.* **269**:19245–19249.
25. Nakamura, H., K. Katayanagi, K. Morikawa, and M. Ikehara. 1991. Structural models of ribonuclease H domains in reverse transcriptases from retroviruses. *Nucleic Acids Res.* **19**:1817–1823.
26. Nakamura, H., Y. Oda, S. Iwai, H. Inoue, E. Ohtsuka, S. Kanaya, S. Kimura, C. Katsuda, K. Katayanagi, K. Morikawa, H. Miyashiro, and M. Ikehara. 1991. How does RNase H recognize a DNA-RNA hybrid? *Proc. Natl. Acad. Sci. USA* **88**:11535–11539.
27. Salazar, M., O. Y. Fedoroff, J. M. Miller, N. S. Ribeiro, and B. R. Reid. 1993. The DNA strand in DNA:RNA hybrid duplexes is neither B-form nor A-form in solution. *Biochemistry* **32**:4207–4215.
28. Skalka, A.-M., and S. P. Goff (ed.). 1993. *Reverse transcriptase*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. Tanese, N., and S. P. Goff. 1988. Domain structure of the Moloney murine leukemia virus reverse transcriptase: mutational analysis and separate expression of the polymerase and RNase H activities. *Proc. Natl. Acad. Sci. USA* **85**:1777–1781.
30. Tanese, N., A. Telesnitsky, and S. P. Goff. 1991. Abortive reverse transcription by mutants of Moloney murine leukemia virus deficient in the reverse transcriptase-associated RNase H function. *J. Virol.* **65**:4387–4397.
31. Telesnitsky, A., S. W. Blain, and S. P. Goff. 1992. Defects in Moloney murine leukemia virus replication caused by a reverse transcriptase mutation modeled on the structure of *Escherichia coli* RNase H. *J. Virol.* **66**:615–622.
32. Temin, H. M., and S. Mizutani. 1970. RNA-directed DNA polymerase in virions of Rous sarcoma virus. *Nature (London)* **226**:1211–1213.
33. Volkmann, S., B. M. Wohrl, M. Tisdale, and K. Moelling. 1993. Enzymatic analysis of two HIV-1 reverse transcriptase mutants with mutation in carboxyl-terminal amino acid residues conserved among retroviral ribonucleases H. *J. Biol. Chem.* **268**:2674–2683.
34. Wintersberger, U. 1990. Ribonucleases H of retroviral and cellular origin. *Pharmacol. Ther.* **48**:259–280.
35. Yang, W., W. A. Hendrickson, R. J. Crouch, and Y. Satow. 1990. Structure of ribonuclease H phased at 2 Å resolution by MAD analysis of the selenomethionyl protein. *Science* **249**:1398–1405.