Vol. 167, No. 1, 1990
February 28, 1990
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 244-250

ENDORIBONUCLEOLYTIC CLEAVAGE OF RNA:OLIGODEOXYNUCLEOTIDE HYBRIDS BY THE RIBONUCLEASE H ACTIVITY OF HIV-1 REVERSE TRANSCRIPTASE

Lindsay R. Dudding, Alexis Harington and Valerie Mizrahi*

Molecular Biology Laboratory, School of Pathology of the South African Institute for Medical Research and University of the Witwatersrand, P.O. Box 1038, Johannesburg 2000, South Africa

Received December 15, 1989

Endoribonucleolytic cleavage by the ribonuclease H activity associated with HIV-1 reverse transcriptase was observed in vitro using substrates consisting of synthetic oligodeoxynucleotides hybridized to a 345 nucleotide T7 RNA polymerase transcript derived from the gag region of HIV-1. This observation suggests that a posssible mechanism of action of antisense oligonucleotides in the inhibition of viral replication and expression may involve the selective "suicidal" ribonucleolytic cleavage of viral RNA by reverse transcriptase at the site of hybridization of the oligonucleotide. © 1990 Academic Press, Inc.

The potential use of antisense oligonucleotides as antiviral agents was originally described by Zamecnik and Stephenson in 1978 (1), and their efficacy was subsequently demonstrated in the inhibition of HIV-1 replication (2,3) and expression (4). Studies on the mechanism by which antisense oligonucleotides hybridized to cognate mRNA molecules result in translational arrest led Walder and Walder to suggest that the predominant mechanism of action of these agents involved ribonucleolytic cleavage of the RNA within the RNA.DNA hybrid region, mediated by cellular RNase H enzymes (5). The RNase H activity associated with HIV-1 RT has been the subject of several recent studies aimed at elucidating the mechanistic features of this

* To whom correspondence should be addressed.

Abbreviations: HIV-1, human immunodeficiency virus type I; AIDS, acquired immune deficiency syndrome; RT, reverse transcriptase; RNase H, ribonuclease H; bp, base pair; nt, nucleotide; Tris.HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

0006-291X/90 $1.50
Copyright © 1990 by Academic Press, Inc.
All rights of reproduction in any form reserved.

key viral function (6-8). The observation of ribonucleolytic cleavage of RNA.DNA hybrid substrates bearing both 3′- and 5′-mismatched RNA termini (8), and of covalently closed hybrids bearing no free RNA termini (7) confirmed that the retroviral RNase H is capable of endoribonucleolytic cleavage *in vitro*. On the basis of these observations, we have studied the *in vitro* RNase H activity of HIV-1 RT using RNA.oligodeoxynucleotide hybrid substrates of defined length and nucleotide sequence to ascertain whether "suicidal" ribonucleolytic cleavage of retroviral RNA could be catalyzed by RT in the presence of antisense oligodeoxynucleotides, thereby providing a possible mechanism of action of these reagents in the inhibition of viral replication and expression.

MATERIALS AND METHODS

Materials. T7 RNA polymerase, RNase inhibitor, *E. coli* RNase H, and pGEM-3Zf(+) were from Promega. T4 polynucleotide kinase and restriction enzymes were from Boehringer-Mannheim. Recombinant HIV-1 RT was isolated from *E. coli* AR120/ pRT1 as described by Mizrahi et al. (9). A gel filtration step was included to ensure complete removal of trace contaminating *E. coli* RNase H. The Q-Sepharose pool was dialyzed against Buffer A/ 0.1 M NaCl/ 1 mM DTT (9). A 3-mL aliquot of the dialysate was concentrated 6-fold (with no activity loss) by centrifugation through two Millipore Ultrafree-MC filter units (30,000 NMWL) in a Millipore Personal Centrifuge (2000 g, 15 min, 4°C). The concentrate was loaded onto a 20-mL Sephadex G-75 column equilibrated in the above buffer, and was eluted at a flow rate of 0.1 mL/min. The recovered RT was >95% pure, and was free of contaminating *E. coli* RNase H as judged by the product profile generated by cleavage of a homogeneous RNA.DNA hybrid substrate, as previously described (8). [α-^{32}P]UTP (600 Ci/mmol) and [γ-^{32}P]ATP (>3000 Ci/mmol) were from Amersham. DE81 filter discs were from Whatman, and Ready-Safe liquid scintillation cocktail was from Beckman. The oligonucleotides P1 (5′-GGTCTACATAGTCTCTAAAA-3′), P2 (5′-CCTGCTATGTCACTTCCCCT-3′), and P3 (5′-TTATCAGAAGGAGCCACCCC-3′) were synthesized using a Beckman oligonucleotide synthesizer and were purified by HPLC prior to use. The subclone pGEM-GAG-C containing the 627 bp *Hin*dIII *gag* fragment of HIV-1 [positions 629-1256; numbering according to Ratner et. al (10)] cloned in a clockwise orientation into the *Hin*dIII site of pGEM-3Zf(+) was previously described (8). The clone pGEM-ΔGAG-C was constructed by deletion of the 341 bp *Pst*I fragment from pGEM-GAG-C.

Methods. (1) T7 transcription. The uniformly labeled transcript (+)-GAG^{345} was prepared by runoff transcription of *Hin*dIII-digested pGEM-ΔGAG-C. Reactions (40 μL) containing 40 mM Tris.HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 1 unit/μL RNase inhibitor, 250 μM each ATP, CTP and GTP, 40 μM [α-^{32}P]UTP (44 Ci/mmol), *Hin*dIII-digested pGEM-ΔGAG-C (0.3 μg/μL), and T7 RNA polymerase (2 units/μL) were incubated at 39-40°C for 2 h. Reactions were quenched by phenol/chloroform (1/1) extraction, followed by ethanol precipitation.

(2) RNA Purification. The precipitate was resuspended in 10 μL TE buffer [10 mM Tris.HCl (pH 8.0)/ 0.1 mM EDTA], and was mixed with an equal volume of denaturing gel electrophoresis sample loading buffer [80% deionized formamide/ 0.1% bromophenol blue/ 0.1% xylene cyanol/ TBE buffer (89 mM Tris base/ 89 mM boric acid/ 2 mM EDTA)], and then loaded onto a 20 cm x 30 cm x 0.8 mm 6% polyacrylamide gel containing 7 M urea in TBE buffer, which was electrophoresed at 400 V for 1 h. The (+)-GAG^{345} RNA was extracted from the gel by agitation in high ionic strength elution buffer [0.5 M ammonium acetate/ 10 mM Mg-acetate/ 1 mM EDTA/ 0.1% SDS] for 18 h at 37°C to avoid the linear acrylamide contamination normally associated with the crush elution procedure (11). The purified RNA was quantitated by measuring the radioactivity remaining on a DE81 filter following a high salt wash (9).

(3) Hybridization. Hybridization conditions between (+)-GAG^{345} and the oligonucleotides P1-P3 were established by non-denaturing gel electrophoretic analysis of hybridization mixtures between unlabeled (+)-GAG^{345} and 5'-end-labeled oligonucleotide. Hybrid formation was only observed between (+)-GAG^{345} and the complementary oligonucleotides P1 and P2, whereas the non-complementary P3 failed to hybridize. For RNase H assays, hybridization mixtures (12.8 μL) containing 40 mM Tris.HCl (pH 7.9), 40 mM KCl, 4 mM $MgCl_2$, uniformly ^{32}P-labeled gel-purified (+)-GAG^{345} (30 nM; $3x10^6$ dpm), and oligonucleotide (P1, P2 or P3; 0.5 μM) were placed in a 500 mL water bath initially at 40-55°C and allowed to cool to 25°C over 1 h.

(4) Gel Electrophoretic RNase H Assay. RNase H assay reactions (25 μL containing 50 mM Tris.HCl (pH 7.9), 50 mM KCl, 7 mM $MgCl_2$, 5 mM DTT, ^{32}P-labeled hybrid (prepared as above) and either HIV-1 RT (12.5 nM) or *E. coli* RNase H (0.04 nM) were incubated at 37°C. Aliquots (4 μL) were withdrawn after 0.5-30 min, quenched with 10 μL sample loading buffer, and 3 μL samples were directly loaded onto a 20 cm x 40 cm x 0.4 mm 8% polyacrylamide gel containing 7 M urea in TBE buffer, and electrophoresed as previously described (8). Gels were autoradiographed at -70°C using 3M Trimax XD film and one intensifier screen.

RESULTS AND DISCUSSION

The subclone used to prepare the T7 runoff transcript (+)-GAG^{345} is illustrated in Figure 1, panel A. The positions of complementarity between (+)-GAG^{345} and the oligonucleotides P1 and P2 are indicated in Figure 1, panel B. The RNase H products formed by HIV-1 RT catalyzed cleavage of the hybrids (+)-GAG^{345}/ P1 and (+)-GAG^{345}/ P2 were analyzed by denaturing gel electrophoresis (Figure 2, panels A and C). For comparison, the *E. coli* RNase H cleavage time courses of these hybrids are illustrated in Figure 2, panels B and D. The lack of cleavage by HIV-1 RT in control reactions containing (+)-GAG^{345} mock-hybridized to either no oligonucleotide, or to the non-complementary oligonucleotide P3 confirmed that the HIV-1 RT was free of contaminating single-strand specific RNases, and

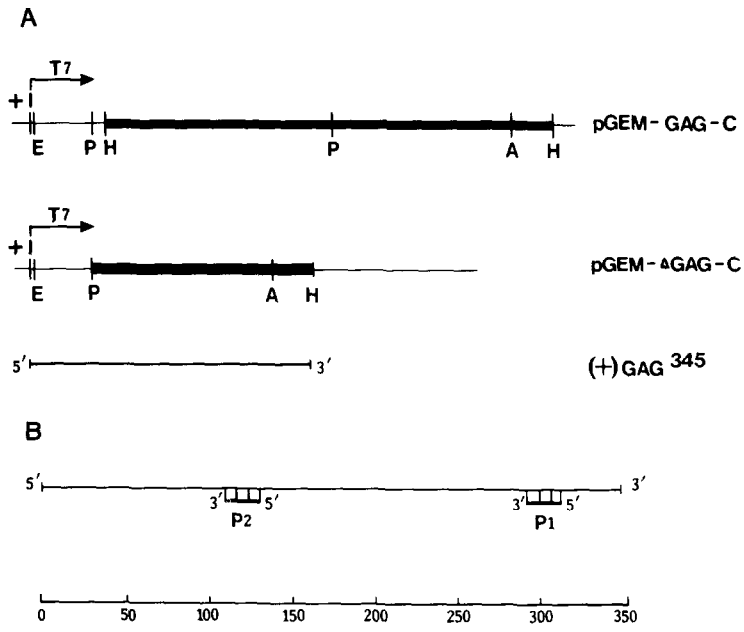

Figure 1. Construction of the RNA.oligonucleotide hybrids. Panel A: Restriction maps of the pGEM gag subclones (additional details described in ref. 8). (+)-GAG^{345} was produced by T7 RNA polymerase runoff transcription of pGEM-ΔGAG-C digested with HindIII (H). Panel B: Location of the regions of complementarity between (+)-GAG^{345} and oligonucleotides P1 and P2.

suggested that the cleavage observed in reactions containing complementary RNA and oligonucleotide components was hybrid-dependent (data not shown). The sizes of the major products were consistent with cleavage exclusively within the base-paired region of (+)-GAG^{345} (Figure 1, panel B). Cleavage of (+)-GAG^{345}/ P2 also yielded minor unexpected 70-75 nt products (Figure 2, panel C) which we tentatively ascribed to hydrolysis of an aberrant 6 bp partial hybrid formed by fortuitous complementarity between P2 and (+)-GAG^{345} (pos. 268-273) (unpublished results). Together, these data suggested that a RNA.DNA duplex structure of at most 20 bp, corresponding to approximately two helical turns, was sufficiently large to allow binding and hydrolysis by the p66/p51 heterodimeric RT enzyme.

Gel electrophoretic analysis of the cleavage products generated by HIV-1 RT and E. coli RNase H indicated significant differences in the RNA.oligonucleotide cleavage site selectivities of the two enzymes, consistent with our previously

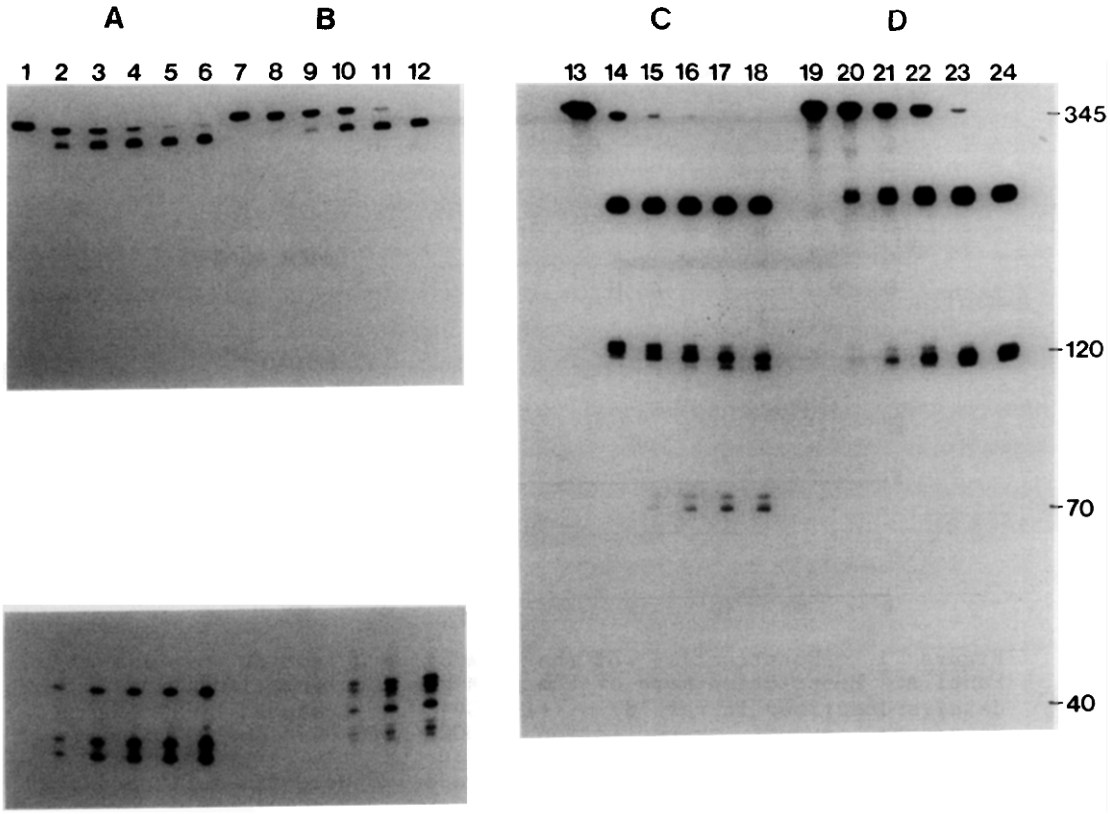

Figure 2. Denaturing gel electrophoretic analysis of RNase H time courses of cleavage of (+)-GAG^{345}/ P1 (panels A and B), and (+)-GAG^{345}/ P2 (panels C and D) catalyzed by HIV-1 RT (panels A and C), and E. coli RNase H (panels B and D), respectively. Reactions were quenched after 0 (lanes 1, 7, 13, and 19), 0.5 (lanes 2, 8, 14, and 20), 5 (lanes 3, 9, 15, and 21), 10 (lanes 4, 10, 16, and 22), 30 (lanes 5, 11, 17, and 23), and 60 min (lanes 6, 12, 18, and 24). Samples were electrophoresed as described under Methods. For the sake of clarity, the 35-45 nt products in panels A and B were visualized by exposure for 35 h compared to 3.5 h for the larger products (>250 nt). Products in panels C and D were visualized by exposure for 12 h. The approximate sizes of the RNA products are indicated.

reported comparative analysis (8). Furthermore, the product size distributions for both enzymes were highly dependent on the initial substrate sequence. Cleavage of (+)-GAG^{345}/ P1 by the 117 kDa HIV-1 RT enzyme resulted in the formation of only three 35-45 nt oligonucleotide products, as opposed to the numerous products generated by the significantly smaller (17.5 kDa (12)) E. coli enzyme (Figure 2, panel A vs. panel B). However, cleavage of (+)-GAG^{345}/ P2 by HIV-1 RT yielded four 115-125 nt products in contrast to the two major E. coli RNase H products (Figure 2, panels C vs. D). Analysis of the time-dependence of the respective product distributions suggested that the 35-45 nt

HIV-1 RT cleavage products of (+)-GAG^{345}/ P1 were all primary, rather than secondary products. In contrast, cleavage of (+)-GAG^{345}/ P2 yielded at least one 115-125 nt secondary product. These differences may be due to the fact that further cleavage of primary hydrolysis products is at least partially determined by the size and sequence (and hence, stability) of the duplex structures remaining after primary RNase H cleavage.

The ability of HIV-1 RT to recognize and endoribonucleolytically cleave a RNA.oligodeoxynucleotide hybrid structure internally contained within a linear RNA molecule adds to the vast catalytic repertoire of this versatile enzyme. The in vivo relevance of this property of HIV-1 RT to the mechanism of action of antisense oligonucleotides and their non-hydrolyzable analogs as therapeutic agents against AIDS, remains to be evaluated. However, given the cytoplasmic location of the RT/ RNase H steps of the normal viral replication cycle (13), suicidal oligonucleotide-directed RNA degradation by RT certainly offers one possible mechanism of action. To facilitate interpretation, the experiments described herein were deliberately conducted in the absence of parallel DNA synthesis by oligonucleotide-primed reverse transcription, which would otherwise compete with the ribonucleolytic reaction under investigation. In this respect, the in vivo effect suggested by our in vitro results possibly may be enhanced by using 3'-dideoxy-terminated oligodeoxynucleotides which are incapable of serving as primers for competitive initiation of DNA synthesis.

Acknowledgments. We are grateful to Professor J. Metz, Dr. C. Debouck and Dr. I. Chaiken for their continued support. This work was funded in part by grants from Smith Kline & French (U.S.A.) and the Goldfields Foundation (S.A.).

REFERENCES

1. Zamecnik, P.C., & Stephenson, M.L. (1978) Proc. Natl. Acad. Sci. USA 75, 280-284.
2. Goodchild, J., Agrawal, S., Civeira, M.P., Sarin, P.S., Sun, D., & Zamecnik, P.C. (1988) Proc. Natl. Acad. Sci. USA 85, 5507-5511.
3. Agrawal, S., Goodchild, J., Civeira, M.P., Thornton, A.H., Sarin, P.M., & Zamecnik, P.C. (1988) Proc. Natl. Acad. Sci. USA 85, 7079-7083.
4. Matsukura, M., Zon, G., Shinozuka, K., Robert-Guroff, M., Shimada, T., Stein, C.A., Mitsuya, H., Wong-Staal, F., Cohen, J.S., & Broder, S. (1989) Proc. Natl. Acad. Sci. USA 86, 4244-4248.

5. Walder, R.Y., & Walder, J.A. (1988) Proc. Natl. Acad. Sci. USA 85, 5011-5015.
6. Starnes, M.C., & Cheng, Y. (1989) J. Biol. Chem. 264, 7073-7077.
7. Krug, M.S., & Berger, S.L. (1989) Proc. Natl. Acad. Sci. USA 86, 3539-3543.
8. Mizrahi, V. (1989) Biochemistry, in press.
9. Mizrahi, V., Lazarus, G.M., Miles, L.M., Meyers, C.A., & Debouck, C. (1989), Arch. Biochem. & Biophys. 273, 347-358.
10. Ratner, L., Haseltine, W., Patarca, R., Livak, K.J., Starcich, B., Josephs, S.F., Doran, E.R., Antoni Rafalski, J., Whitehorn, E.A., Baumeister, K., Ivanoff, L., Petteway, S.R., Pearson, M.L., Lautenberger, J.A., Papas, T.S., Ghrayeb, J., Chang, N.T., Gallo, R.C., & Wong-Staal, F. (1985) Nature 313, 277-284.
11. Ogden, R.C., & Adams, D.A. (1987) Methods Enzymol. 152, 61-87.
12. Kanaya, S., & Crouch, R.J. (1983) J. Biol. Chem. 258, 1276-1281.
13. Haseltine, W.A. (1989) J. Acq. Immun. Defic. Syndromes 2, 311-334.