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Interaction of HIV-1 Ribonuclease H with Polypurine Tract Containing RNA–DNA Hybrids†

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ABSTRACT: The mode of action of the RNase H activity from HIV-1 was analyzed with a purified recombinant p66/p51 reverse transcriptase RT/RNase H protein and RNA–DNA hybrid consisting of RNA harboring the polypurine tract (ppt) and three complementary synthetic DNA oligonucleotides. Upon incubation of this preformed RNA–DNA hybrid with the p66/p51 RT/RNase H, a cleavage pattern is observed that indicates endonucleolytic RNase H activity with some sequence specificity for the next to last nucleotide of the 3′-end of the ppt RNA and one cut within the ppt. The RNase H avoids cleavage of G or A stretches. During RNA-directed DNA synthesis the RNA is hydrolyzed in a concerted action of RT and RNase H whereby the RNase H exhibits endonuclease as well as 3′–5′-exonuclease activity. The distance between the active centers of the RT and RNase H corresponds to 18 base pairs of the RNA–DNA hybrid. Plus-strand DNA-directed DNA synthesis initiates exactly at the next to last nucleotide of the 3′-end of the ppt RNA by means of the RNase H activity.

Reverse transcription of retroviral RNA to DNA involves RNA-directed as well as DNA-directed DNA synthesis studied in various in vitro systems (Gilboa et al., 1979). The synthesis of the first DNA strand is initiated at a tRNA primer and proceeds by copying viral RNA. In the course of the minus-strand DNA synthesis the genomic RNA is degraded (Collett et al., 1978). This degradation allows binding of the minus-strand DNA to the 3′-end of the viral genome via the terminal redundancies (R) (for illustration, see Figure 1). Minus-strand DNA synthesis can be completed while plus-strand DNA synthesis needs to be initiated at a newly formed primer presumably at the 3′-end of the polypurine tract (ppt), a highly conserved sequence present in all 3′-regions of retroviral RNAs (Resnick et al., 1984; Rattray & Champoux, 1989). The final product is a duplex DNA free of RNA. All known reverse transcriptases (RTs) show two catalytic activities, a DNA polymerase activity and an associated RNase H activity specific for the degradation of RNA in RNA–DNA hybrids (Moelling et al., 1971; Moelling, 1974; Verma, 1975, 1977; Gerard & Grandgenett, 1975; Hansen et al., 1987). The RNase H generates the polypurine-rich oligoribonucleotide (ppt) that primes synthesis of plus-strand DNA (Resnick et al., 1984; Champoux et al., 1984; Finston & Champoux, 1984; Smith et al., 1984a,b). The RT from HIV particles has been shown to be present as a 66/51-kDa heterodimer with a common amino terminus (Di Marzo Veronese et al., 1986; Lowe et al., 1988). The RNase H domain is located at the carboxy terminus of p66 (Johnson et al., 1986; Hansen et al., 1988). Similarly, a p66/p51 heterodimeric protein can be expressed by recombinant DNA technology in *Escherichia coli* and exhibits similar functions (Farmerie et al., 1987; Hansen et al., 1988; Mous et al., 1988).

The RNase H activities of reverse transcriptases were believed to be exonucleases that can attack RNA from either the 5′- or the 3′-end, whereas the normal cellular RNase H activities are endonucleases (Keller & Crouch, 1972; Leis et al., 1973; Moelling, 1974; Büsen, 1980). Recently, Krug and Berger (1989) and Oyama et al. (1989) analyzed various model hybrids that were cleaved by the RNase H of avian, murine, and human retroviruses in an endonucleolytic manner. Furthermore, the two primers, the tRNA primer for the minus-strand DNA and the ppt RNA for the plus-strand DNA synthesis, seem to be released by an endonucleolytic mecha-

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nism (Omer & Faras, 1982; Champoux et al., 1984). The ppt sequence requirements for the murine viral RNase H have been analyzed by mutations (Rattray & Champoux, 1989).

Here we set out to characterize the mode of action of the HIV-1 RNase H in the ppt region and determine if it is specifically cleaved by an endonucleolytic RNase H. Furthermore, our results demonstrate a concerted action between RT and RNase H during RNA-directed DNA synthesis. While this work was in progress, Schatz et al. (1990) described also a concerted HIV-1 RT/RNase H action using random RNA–DNA hybrids. Luo et al. (1990) and Huber and Richardson (1990) presented evidence that AMV and HIV-1 RNase H specifically make use of the ppt RNA for plus-strand DNA synthesis.

Experimental Procedures

Materials. Restriction enzymes, bacterial alkaline phosphatase, and the vanadyl ribonucleoside complex (VRC) were purchased from BRL, Berlin, West Germany. *Hae*III-restricted pBR322 DNA, T4 polynucleotide kinase, T4 DNA ligase, RNase-free DNase I, *E. coli* RNase H, 2′,3′-dideoxynucleoside triphosphates (ddNTPs), and 2′-deoxynucleoside triphosphates (dNTPs) were from Boehringer Mannheim, Mannheim, West Germany. RNasin was from Promega Biotech, Heidelberg, West Germany. Sequencing reactions were done with the Sequenase kit from USB, Cleveland, OH. AMV RT and the vector pTZ19R harboring the T7 promoter were from Pharmacia, Uppsala, Sweden. Radiolabeled compounds [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]UTP (>400 Ci/mmol), [α-^{35}S]dATPαS (>1000 Ci/mmol), and [^{3}H]TTP (42 Ci/mmol) were from Amersham Buchler, Braunschweig, West Germany. The RNA transcription kit, T7 RNA polymerase, and AMV reverse transcriptase sequencing kit were from Stratagene, Heidelberg, West Germany. DNA oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer and were purified by HPLC prior to use.

DNA Manipulations. Restriction endonuclease cleavage, DNA isolation, ligation, end labeling of DNA, and transformations were performed as described in Maniatis et al. (1982).

Plasmid Constructions. The 474 base pair long *Pvu*II fragment from the HIV-1 provirus clone BH10 (kindly supplied to us by Dr. R. C. Gallo) including the polypurine tract (ppt) was cloned into the *Sma*I site of the T7 transcription vector pTZ19R to yield plasmid pTZP8, which was further used in RNA transcription assays. The same *Pvu*II fragment was cloned in both orientations into DNA of the phage M13mp18. For isolation of DNA the constructs were transformed into the *E. coli* K12 strain JM109 (Yannisch-Perron et al., 1985).

In Vitro Transcription with Plasmid pTZP8. A total of 1 μg of DNA of plasmid pTZP8 was linearized with *Eco*RI and, after proteinase K treatment and phenol/chloroform extraction, was transcribed by T7 RNA polymerase according to the conditions described in the Stratagene RNA transcription kit. Thus an RNA of 534 bases in length was synthesized including the ppt and flanking regions. High specific RNA probes were uniformly labeled with 40 μM UTP and 50 μCi of [α-^{32}P]UTP, and low specific RNA was obtained with 200 μM UTP and 50 μCi of [α-^{32}P]UTP.

Purification of HIV-1 RT/RNase H. The bacterially expressed recombinant RT/RNase H was purified as described previously with a poly(U)–Sepharose instead of an Aga(rC) column (Hansen et al., 1987, 1988). One unit of purified HIV-1 RT/RNase H catalyzes the incorporation of 13 pmol of [^{3}H]TMP into acid-insoluble products in 10 min at 37 °C with poly(rA)·(dT)$_{10}$ as template/primer. The concentration of the enzyme corresponds to about 1 μL per unit.

Sequencing Reactions. Sequencing reactions were carried out by using the dideoxy sequencing kit from USB according to the manufacturer's instructions except with a ^{32}P-end-labeled oligonucleotide primer in some reactions. Aliquots were analyzed on 8% or 10% TBE–urea–polyacrylamide gels.

RNase H Cleavage of RNA–DNA Oligonucleotide Hybrids. Unless otherwise stated standard reactions were carried out in a total volume of 20 μL in RNase H buffer (50 mM Tris-HCl, pH 7.8, 40 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT) containing 20 units of RNasin. A total of 0.2 unit of HIV-1 RT/RNase H, 3 units of AMV RT/RNase H, or 4 units of RNase H from *E. coli* was added. (Unit definitions of AMV RT and RNase H from *E. coli* are according to the descriptions given by the manufacturers.) Reactions were started by the addition of 0.2 pmol of the RNA (high specific activity) prehybridized in the presence of a 20–25-fold molar excess of the corresponding DNA oligonucleotide. The mixture was incubated for 30 min at 37 °C, and the reactions were terminated by the addition of 6 μL of urea loading buffer (7 M urea in 1× TBE, 0.1% each xylene cyanol and bromophenol blue). Aliquots were analyzed by electrophoresis on a 10% TBE–urea–polyacrylamide gel.

RNase H Reaction Coupled with cDNA Elongation. To the standard reaction mixture described above were added a 125 μM quantity of one of the ddNTPs and each of the other three dNTPs and 4 units of HIV-1 RT. The reactions were started by the addition of approximately 3 pmol of hybrid RNA (low specific activity) and treated as described above. To check for cDNA elongation, the same test was carried out, except with unlabeled in vitro transcribed RNA hybridized to a 10:1 mixture of unlabeled to labeled oligonucleotide. Aliquots were analyzed by electrophoresis on 10% TBE–urea–polyacrylamide gels.

RNase H Reaction and Oligonucleotide Extension Assays of RNase H Treated Model Substrates X, Y, and Z. The RNase H reaction was carried out in a total volume of 500 μL in RNase H buffer containing 1000 units of RNasin and 25 units of HIV-1 RT/RNase H. After the reaction was started by the addition of 8 pmol of an unlabeled RNA–DNA oligonucleotide hybrid, the sample was divided into five aliquots of 100 μL and incubated for 30 min at 37 °C, and the reaction was stopped at 95 °C for 5 min. The samples were placed on ice and to each was added 10 units of DNase I to remove the DNA oligonucleotide. After 10 min the samples were incubated further at 37 °C for 10 min. The reactions were stopped at 95 °C for 10 min. A ^{32}P-end-labeled DNA oligonucleotide primer N complementary to a sequence 66–86 bases downstream of the ppt was annealed to the cleaved RNA and the primer extended up to the RNase H cleavage site in a total volume of 15 μL in 1× RT buffer (Stratagene sequencing kit) by means of 7 units of AMV RT (Stratagene sequencing kit), 20 units of RNasin, and dNTPs to 330 μM. After 1 h at 42 °C the reaction was stopped with 5 μL of formamide buffer included in the kit. Aliquots were analyzed by electrophoresis on an 8% TBE–urea–polyacrylamide gel together with dideoxy sequencing ladders generated with the same ^{32}P-end-labeled DNA oligonucleotide primer on a single-stranded M13 DNA template harboring the corresponding DNA fragment.

RNase H Reaction and Reverse Transcription Using an M13 RNA Hybrid as Substrate. A total of 2.5 μg of in vitro transcribed RNA and 8 μg of the M13 single-stranded DNA harboring the complementary fragment were hybridized in RNase H buffer in a total volume of 25 μL by heating the

mixture to 80 °C and cooling it slowly (1–2 h) to room temperature. The RT/RNase H reaction was carried out in a total volume of 50 μL in RNase H buffer containing 50 units of RNasin, 10 units of HIV-1 RT/RNase H, and a 250 μM quantity of each dNTP. The reaction was started by the addition of 25 μL of the hybridization mixture, the mixture was incubated for 40 min at 37 °C, and the reaction was terminated by heating the sample to 95 °C for 3 min. After treatment with 0.3 M NaOH at 65 °C for 20 min, ammonium acetate (pH 4.8) was added to 3 M, and the products were precipitated with 4 volumes of absolute ethanol and washed three times with 70% ethanol to remove salt and free nucleotides. An oligonucleotide extension assay was carried out with the ^{32}P-end-labeled DNA oligonucleotide N described above in 1× Sequenase buffer (Sequenase kit) in a total volume of 20 μL. The mixture contained 5 mM DTT, a 250 μM quantity of each dNTP, and 7.5 units of Sequenase and was incubated for 40 min at 37 °C. The reaction was stopped by the addition of 5 μL of formamide buffer included in the Sequenase kit. Electrophoresis was performed on a 10% gel along with dideoxy sequencing ladders generated on the corresponding M13 DNA template with the unlabeled DNA primer N and [α-^{35}S]dATPαS.

Results

First we set out to investigate the RNase H activities involved in cleavage of preformed hybrids containing the ppt sequence. The corresponding DNA of the ppt RNA and that of the flanking RNA sequences from the 3′-end of the BH10 DNA provirus were cloned into the T7 transcription vector pTZ19R. Three DNA oligonucleotides ranging from 20 to 40 nucleotides, complementary to the ppt RNA and flanking sequences as indicated in Figure 1, were used to form model hybrid substrates designated as X, Y, and Z. The RT used was expressed by recombinant protein in *E. coli* as a p66/p51 heterodimer from a ptrp9-pol construct described previously (Hansen et al., 1987, 1988). RNA affinity chromatography removes RNase H activity of *E. coli* origin, confirmed by glycerol density gradient centrifugation (Hansen et al., 1988; data not shown). The HIV-1 p15 RNase H was undetectable by immunoblotting using an RNase H specific monoclonal antibody (data not shown). The RNase H was inhibited by vanadyl ribonucleoside complexes (VRC) proving its viral origin (Krug & Berger, 1986, 1989; data not shown).

Various RT/RNase H concentrations were allowed to react with radioactively labeled RNA hybridized to the three DNA oligonucleotides X, Y, and Z (Figure 2A). The resulting reaction products analyzed on a 10% polyacrylamide gel indicate discrete preferential endonucleolytic cleavage sites for all three substrates. Two prevailing cleavage sites are localized around nucleotide 79 at the 3′-end of the ppt RNA and at nucleotide 72 close to the middle of the ppt RNA, based on the sequencing analysis shown in Figure 2C (see below). In the case of the shortest hybrid X cleavage occurs only around the 3′-end of the ppt, probably because there is no space for the RT/RNase H molecule to allow a second cut closer to the 5′-end. At highest enzyme concentrations all of the hybrid was cleaved and some shorter 5′-RNA molecules of 60 and 62 bases in length show up. While the two larger 5′-RNA molecules arise by endonucleolytic cuts, the shorter ones may involve additional processive 3′–5′-exonuclease activity. The processive nature of the RNase H in the absence of DNA synthesis has been described before (Hansen et al., 1988). The RNase H apparently prefers cleavage at two specific sites avoiding especially homopolymeric stretches of A and G, which are apparently more resistant to cleavage than heteropolymeric

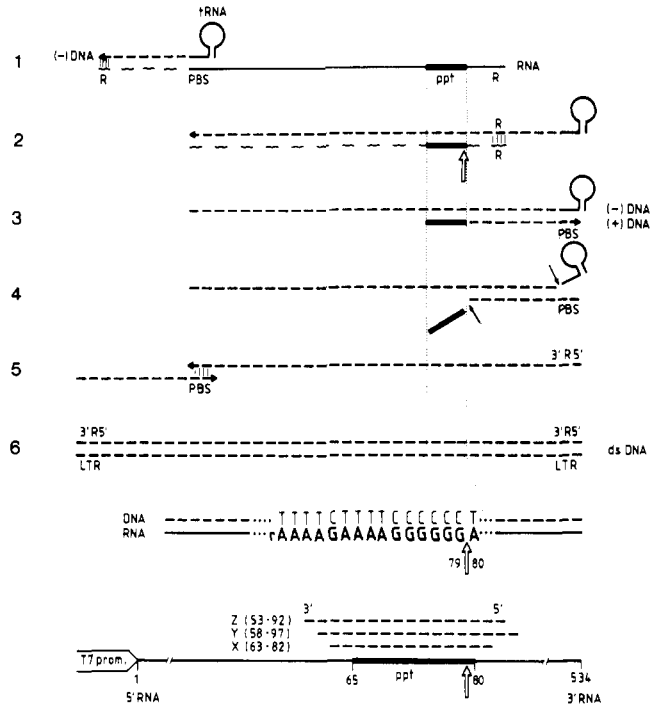

FIGURE 1: Reverse transcription of RNA into double-stranded DNA. Minus-strand RNA-directed DNA synthesis initiates at the tRNA primer (stage 1) and according to this study can occur simultaneously with removal of the template RNA by the RNase H (stages 1 and 2), whereby a ppt RNA primer needs to be conserved to allow initiation of plus-strand DNA-directed DNA synthesis (stages 2 and 3). For details, see text. The model hybrid substrates X, Y, and Z used in this study are illustrated. Abbreviations: PBS, primer binding site; ppt, polypurine tract; R, terminal redundancy; ds, double strand; LTR, long terminal repeat. Symbols: RNA (—), DNA (– – –), and hydrolyzed RNA (~).

regions. Figure 2B illustrates the effect of salt on the RNase H activity and the three model substrates X, Y, and Z. The patterns obtained resemble those received by titration of the RNase H shown in Figure 2A, allowing a similar interpretation. It appears that at salt concentrations above 120 mM sodium chloride the endonuclease activity predominates whereas the exonuclease activity is impaired. A final concentration of 60 mM salt was used for further studies. Figure 2C shows the sequencing experiment performed to determine the cleavage site of hybrid X by the RNase H.

Concerted Action of RT and RNase H. In order to analyze the mode of action of the RNase H in the presence of DNA synthesis, an experimental approach was designed which allowed primer extension by action of the RT and simultaneous analysis of the RNA. A defined primer extension was achieved by the addition of three deoxyribonucleotides (dNTPs) and one dideoxyribonucleotide (ddNTP), which leads to chain termination (Sanger et al., 1977). In four independent reactions ddATP, ddGTP, ddTTP, or ddCTP was added to model substrate X and allowed—in addition to RNA cleavage—extension of the DNA oligonucleotide by the RT by +1, +4, +5, and +8 deoxyribonucleotides, respectively. Analysis of the resulting 5′-RNA fragments is shown in Figure 3A (left) and indicates that after an endonucleolytic cut (at ribonucleotide 80/81) has taken place, the RNase H proceeds with a 3′–5′-exonuclease activity by hydrolyzing −1, −4, −5, and −8 ribonucleotides, respectively. The RNase H cleavage sites are accompanied by other less frequently occurring cuts, indicating some imprecision of the RNase H activity. Use of the model substrate Y leads to similar results (Figure 3A, right), whereby the endonucleolytic cut takes place at nucleotide 75/76, not 79, the site preferred in the absence of

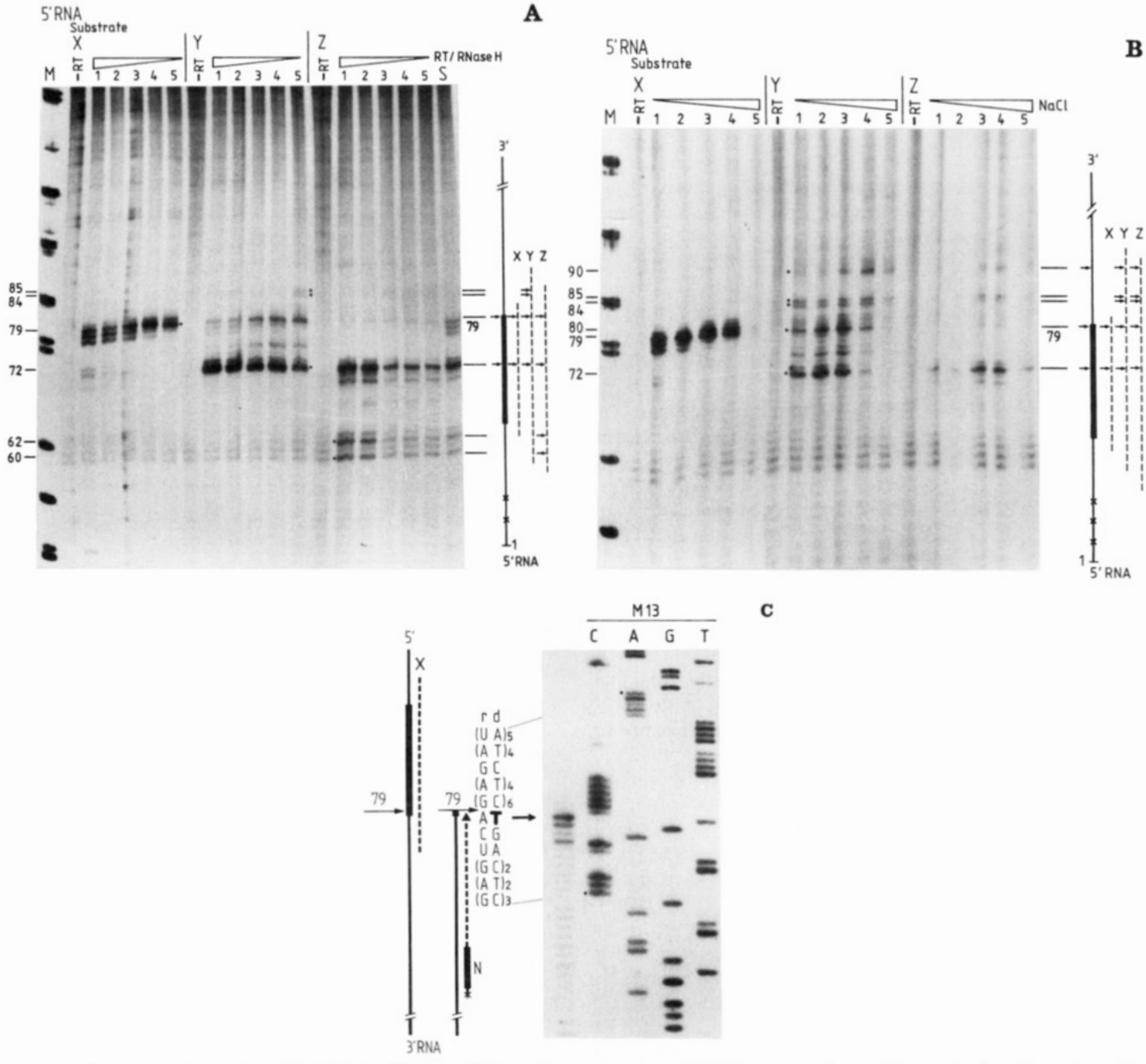

FIGURE 2: Cleavage patterns of model substrates X, Y, and Z depending on enzyme and NaCl concentrations and determination of a preferential cleavage site. (A, B) Hybrids X, Y, and Z were treated with 4, 1, 0.25, 0.125, and 0.0625 unit, respectively (panel A, lanes 1–5, X, Y, Z), of HIV-1 RT/RNase H in the standard reaction mixture or with 0.2 unit of HIV-1 RT/RNase H and final NaCl concentrations of 20, 60, 100, 180, and 220 mM (panel B, lanes 1–5, X, Y, Z) at 37 °C for 30 min. The resulting 5′-RNA fragments were separated on 10% sequencing gels. The numbers on the left indicate the size of the 5′-RNA fragments (see Figure 1, bottom); the arrows on the right point to the cleavage sites in hybrids X, Y, and Z. Nucleotide 79 corresponds to the 3′-end of the ppt RNA. −RT indicates incubation of hybrid without enzyme; S (panel A) represents a combination of the reactions shown in lanes 1 of each of the hybrids X, Y, and Z; M represents *Hae*III fragments of pBR322 as molecular weight markers. An asterisk indicates radioactive labeling of the RNA. (C) Determination of the cleavage site in hybrid X. A total of 8 pmol of hybrid X was incubated with 25 units of HIV-1 RT/RNase H. The resulting 3′-RNA cleavage product was hybridized to the ^{32}P-end-labeled DNA oligonucleotide N, and a primer extension assay was performed with AMV RT to determine the 5′-end of the 3′-RNA fragment (position 79). An aliquot of the reaction product was applied on an 8% sequencing gel together with the sequence ladder of a single-stranded M13 DNA harboring the corresponding plus-strand fragment. Lanes C, A, G, and T contain the sequencing products of the M13 DNA with the same priming oligonucleotide N.

DNA synthesis (compare slot 3 with 5 and slot 11 with 13). The DNA-polymerizing domain of the RT apparently forces the RNase H to cleave at a distance 18 base pairs behind the DNA synthesis initiation site. This distance appears to reflect the distance between the active centers of the RT and RNase H molecule. Since the two DNA oligonucleotide primers in substrates X and Y are longer than 18 nucleotides, the RNase H endonucleolytic cleavage site does not coincide with the preferred ppt cleavage site at nucleotide 79, cleaved in the absence of DNA synthesis.

To prove that extension of the DNA had actually taken place, conditions were chosen such that the DNA oligonucleotide instead of the RNA strand of model substrate Y was radioactively labeled and extended by the action of the RT in the presence of ddGTP, ddCTP, ddTTP, and ddATP in analogy to the approach described for Figure 3A. Analysis of the extended DNA oligonucleotides is demonstrated in Figure 3B. As a control, primer extension in the absence of chain terminator nucleotides is shown in lane 2, which leads to a DNA copy of 97 nucleotides corresponding to the complete 5′-RNA (compare with Figure 1).

Polypurine Tract Primer Generated for Plus-Strand DNA Synthesis. The ppt RNA is anticipated to play a role as primer in plus-strand DNA synthesis at a later stage of the replication

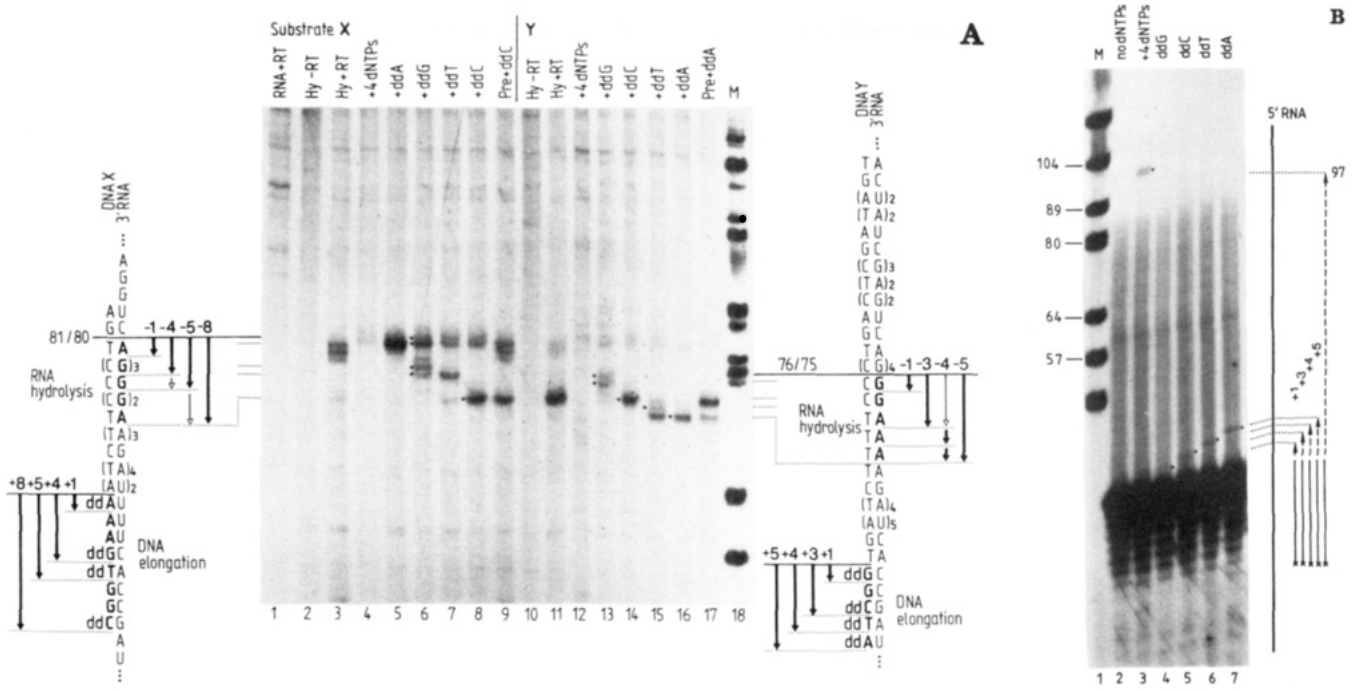

FIGURE 3: Concerted action of RT and RNase H during RNA-dependent DNA synthesis. (A) RNase H reactions were carried out with RNA–DNA hybrids X and Y labeled with low specific activities with 4 units of HIV-1 RT/RNase H and 3 pmol of hybrid in 20 μL at 37 °C for 30 min in the absence or presence of three dNTPs and one ddNTP, and the 5′ products were separated on a 10% sequencing gel. Lane 1, RNA incubated with HIV-1 RT/RNase H; lanes 2 and 10, hybrid without enzyme; lanes 3 and 11, cleavage products obtained by RT/RNase H in the absence of all nucleotides; lanes 4 and 12, cleavage products obtained in the presence of RT/RNase H and four dNTPs; lanes 5–8 and 13–16, incubation in the presence of one ddNTP as indicated and the other three dNTPs; lanes 9 and 17 (Pre + ddN), products obtained after preincubation in the absence of dNTPs and ddNTPs followed by treatment with one ddNTP and the other three dNTPs; lane 18 (M), *Hae*III fragments of pBR322 as molecular weight markers. The numbers 81/80 and 76/75 represent the nucleotides of the cleavage sites and the start points of RNA hydrolysis. The nucleotides incorporated during DNA elongations and concomitant RNA hydrolysis are indicated. (B) Evidence for cDNA elongation. RT/RNase H reactions were carried out as in (A) with hybrid Y except with unlabeled RNA and ^{32}P-end-labeled DNA oligonucleotide in the hybrid. DNA products were separated on a 10% sequencing gel. DNA products obtained by RT in the absence or presence of four dNTPs are shown in lanes 2 and 3, respectively. Lanes 4–7 show DNA products obtained by RT in the presence of one ddNTP as indicated and the other three dNTPs. The number of nucleotides incorporated during cDNA elongation is shown on the right; 97 indicates the number of bases of the full-length DNA copy (see also Figure 1, substrate Y). Lane 1 (M) represents *Hae*III fragments of pBR322 as molecular weight markers.

cycle (see Figure 1). At this step a sequence-specific endonucleolytic cut involving the ppt is expected to occur by the viral RNase H. In order to reproduce this process in vitro, a new model substrate was conceived consisting of RNA containing the ppt sequence and flanking sequences and of single-stranded complementary M13 DNA (Figure 4). By the addition of all four deoxyribonucleotides, action of the RT and possibly the RNase H is allowed. The cleavage site was determined by primer extension of oligonucleotide N between nucleotides 79 and 80, the next to last nucleotide of the 3′-end of the ppt. This result demonstrates that the RT/RNase H specifically generates a ppt RNA primer for plus-strand DNA synthesis. Two mechanisms are possible. The primer can arise either by a ppt sequence-specific endonucleolytic RNase H cleavage or by a 3′–5′ processive exonucleolytic RNase H which leaves the ppt RNA primer, shortened by one nucleotide, otherwise intact.

Discussion

We have analyzed the specificity of the HIV-1 RT-associated RNase H with ppt-containing hybrids during the absence of DNA synthesis and during RNA-directed and DNA-directed DNA synthesis. During the absence of DNA synthesis the RNase H preferentially cleaves at two sites, at the next to last nucleotide of the 3′-end of the ppt (nucleotide 79, see Figure 1) and seven bases further within the ppt (nucleotide 72) (Figure 2). This second cleavage is apparently not possible with the shortest DNA oligonucleotide, supposedly because of steric reasons. Other cleavage sites outside of the ppt region were also observed, indicating that the sequence specificity of the RNase H is not absolute. Selection of the correct primer occurs with initiation of plus-strand DNA synthesis (see Figure 4). While cleavage at nucleotide 79 in substrate X is clearly achieved by an endonuclease, the other cleavage sites could originate either by a series of endonucleolytic cuts or by an exonucleolytic RNase H activity. Processive exonuclease activity has been described for the retroviral RT/RNase H enzymes (Moelling, 1974; Hansen et al., 1988). The cleavage pattern obtained indicates that the RNase H does not produce mononucleotides as reaction products but rather exhibits a preference for cleavage between heteroribonucleotides especially avoiding A and G clusters. Luo et al. (1990) performed a study with avian and murine retroviral RNase H activities in which a 12 base long ppt sequence is shown to serve as the primer for plus-strand DNA synthesis. The ppt tract in the case of HIV-1 is longer. Either the complete ppt or the seven bases observed at nucleotide 72 could serve as the primer. This residue is an important one according to Rattray and Champoux (1989), who concluded from mutational analysis of the ppt of murine viruses that the A residue at the −7 position from the RNase H cleavage site is critical for positioning the RNase H for correct cleavage. The distance between the active sites of RT and RNase H, corresponding to 18 nucleotides, suggests that the complete ppt, which is 16 nucleotides long, could serve as primer.

The RNase H clearly functions as an endonuclease in this assay, confirming what has recently been described (Krug & Berger, 1989). We and others have formerly characterized

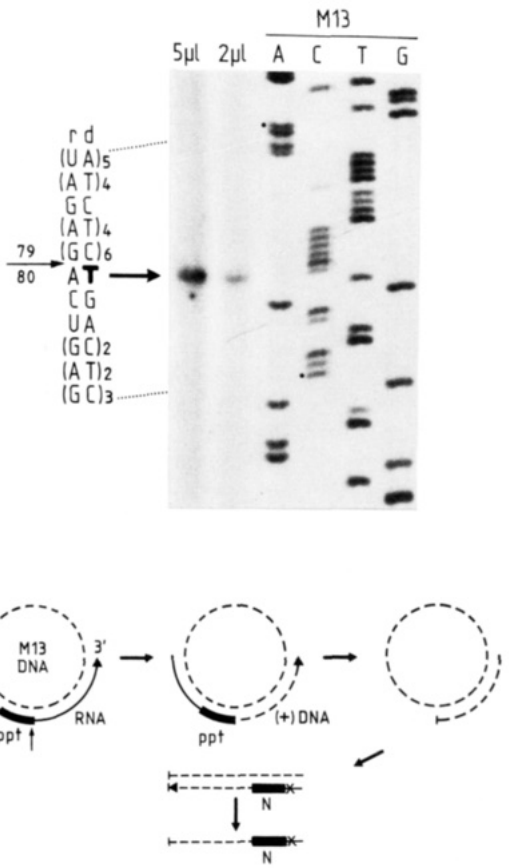

FIGURE 4: Determination of the preferential cleavage site of the start of plus-strand DNA synthesis. DNA oligonucleotide extension assay. An M13 DNA–RNA hybrid harboring the ppt region was incubated with RT/RNase H and four dNTPs, which leads to (+) DNA synthesis by the RT-specific DNA-directed DNA polymerase. To determine the initiation site of the (+) DNA, first the RNA was removed by alkali. A ^{32}P-end-labeled oligonucleotide N was annealed to the (+) DNA and used for a primer extension reaction with Sequenase as the polymerizing enzyme. Two aliquots (2 and 5 μL) of the reaction product were applied to an 8% sequencing gel. Lanes A, C, T, and G contain the products of dideoxy sequencing reactions carried out with single-stranded M13 DNA, harboring the corresponding plus-strand DNA fragment and oligonucleotide N as the primer.

the retroviral RNase H activities as processive exonucleases (Keller & Crouch, 1972; Moelling, 1974). The basis for this conclusion was a special substrate consisting of supercoiled double-stranded ColE1 DNA with short RNA inserts—the only hybrid available then. Such a substrate may not allow the action or detection of an endonucleolytic RNase H.

Under conditions that allow RNA-directed DNA synthesis to occur in a DNA oligonucleotide primed reaction, the RNase H exhibits both, endo- and exonuclease activity (Figure 3). In such a reaction elongation of the DNA by one to eight deoxynucleotides is exact, whereas the RNase H appears to be less precise and seems to slide over or avoid cleavage of homonucleotide clusters (Figure 3). The RT and RNase H can function in a concerted action—a mechanism that has been described for the HIV-1 RNase H for unspecific hybrids by Schatz et al. (1990), for AMV by Oyama et al. (1990), and for HIV by Huber and Richardson (1990). The RNase H cleavage site in this reaction is dictated by the initiation site of DNA synthesis and takes place 18 nucleotides upstream. This distance apparently reflects the space between the active centers of the RT and RNase H domains. This distance may depend to some extent on the local RNA sequence as was discussed by Oyama et al. (1989), who observe a 7–14 nucleotide long distance between RT and RNase H of AMV. Schatz et al. (1990) describe a distance of 7 nucleotides with an unspecific hybrid.

The concerted RT/RNase H mechanism could apply to two stages of the model shown in Figure 1—synthesis of the strong-stop minus-strand DNA (stage 1) and completion of the minus-strand DNA (stage 2). The model for strong-stop DNA is often drawn as two separate steps (Stryer, 1988). Our data suggest that this can occur simultaneously.

In a third approach we designed a model substrate that would mimic the initiation of plus-strand DNA synthesis (Figure 4). The ppt sequence is specifically recognized by the RT/RNase H in this assay, and cleavage occurs exactly at the next to last nucleotide of the 3′-end of the ppt RNA. Our result confirms what has been predicted from other retroviral studies (Mitra et al., 1982; Champoux et al., 1984; Resnick et al., 1984; Rattray & Champoux, 1987; Lou et al., 1990), especially from the comparative analysis by Rattray and Champoux (1989) and the recent study by Huber and Richardson (1990), who included the HIV-1 ppt. The ppt RNA can be generated either by an endonucleolytic ppt-specific RNase H cut or by an exonucleolytic RNase H that removes the RNA from the 3′-end, avoiding the ppt sequences shortened by one nucleotide before DNA synthesis initiates. During the viral life cycle the ppt primer may alternatively be generated already during minus-strand DNA synthesis coupled with RNase H (see stage 2, Figure 1). Our experiment proves that the RNase H recognizes the ppt sequence with high specificity and allows initiation of plus-strand DNA synthesis exactly at the predicted site (Rattray & Champoux, 1989).

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Articles

Melting and Chemical Modification of a Cyclized Self-Splicing Group I Intron: Similarity of Structures in 1 M Na^+, in 10 mM Mg^{2+}, and in the Presence of Substrate†

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ABSTRACT: C IVS is the cyclized form of the intron from the RNA precursor of the *Tetrahymena thermophila* large subunit (LSU) ribosomal RNA. C IVS was mapped by chemical modification in 1 M Na^+, 0.05 M Na^+ and 10 mM Mg^{2+} (Na^+/Mg^{2+}), and Na^+/Mg^{2+} with CUCU substrate. The results suggest the secondary structure is similar for all three conditions. Optical melting curves were also measured for C IVS in 1 M Na^+ and Na^+/Mg^{2+} and indicate the secondary structures have similar stabilities under both conditions. Computer predictions of secondary structure and stability are in good agreement with observations. The results suggest that many of the approximations used for computer prediction of secondary structure by free energy minimization are reasonable.

Free energy minimization methods with a nearest-neighbor model predict about 70% of RNA secondary structure from sequence (Jaeger et al., 1989). To improve predictions, it is necessary to know the validity of assumptions in the current model. One assumption is that free energy parameters measured in 1 M NaCl (Borer et al., 1974; Freier et al., 1986; Turner et al., 1988) are relevant for predicting structures formed in the presence of Mg^{2+}. Another assumption is that secondary structure dominates the free energy of folding so that tertiary interactions can be neglected (Tinoco et al., 1971; Papanicolaou et al., 1984; Jaeger et al., 1989). Studies of the folding of tRNAs are consistent with these assumptions (Cole et al., 1972; Riesner & Römer, 1973; Crothers et al., 1974). Most RNAs, however, are more complex than tRNA. For example, tRNAs have no bulges, internal loops, or pseudoknots (Rietveld et al., 1982; Pleij et al., 1985). Thus, it is important to test these assumptions in other RNAs. C IVS is the cyclized form of the intron from the RNA precursor of the *Tetrahymena thermophila* large subunit (LSU) ribosomal RNA (Zaug et al., 1983). This work uses C IVS to test assumptions in free energy minimization methods by comparing chemical modification patterns and thermal melting profiles in 1 M NaCl and in 0.05 M Na^+/10 mM Mg^{2+} and by comparing these results to computer prediction. This system is also used to test whether substrate binding affects RNA folding.

The *T. thermophila* LSU intron was the first known self-splicing RNA (Kruger et al., 1982). Its structure has been studied extensively by phylogenetic analysis (Michel & Dujon, 1983; Warring & Davies, 1984; Cech, 1988), mutagenesis [see Burke (1988) for a recent review], enzymatic digestion (Cech et al., 1983; Been et al., 1987), and chemical modification (Inoue & Cech, 1985; Latham & Cech, 1989). On the basis

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