Reverse transcriptase reads through a 2'-5' linkage and a 2'-thiophosphate in a template

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ABSTRACT

Avian myeloblastosis virus and Maloney murine leukemia virus RNase H + reverse transcriptases pause when they encounter a 2'-5' linkage or a 2'-thiophosphate in their template RNAs, but eventually read through these backbone modifications. Both reverse transcriptases pause after the 2'-5' linkage but before the 2'-thiophosphate. These results suggest that in the absence of precise information concerning the behavior of a given reverse transcriptase with respect to a particular lesion or modification, caution should be exercised in the interpretation of primer extension data that is being used to determine the existence of, or map the position of, a crosslink, site of chemical modification or non-standard linkage in an RNA template.

INTRODUCTION

Reverse transcriptase is frequently used to determine the position of RNA modifications produced by chemical reagents, ultraviolet light or enzymes. The interpretation of primer extension analysis relies on knowledge of the behavior of reverse transcriptase when it encounters a particular kind of modification. When reverse transcriptase encounters an altered nucleotide on a template strand it may read through, pause or terminate; continued primer extension may be accomplished with fidelity or with the insertion of a random or particular incorrect base, while pausing and termination may occur before, at or after the modified site. The behavior of several reverse transcriptases when they encounter abasic sites (1,2), N2-methylguanines (3), UV and chemically induced crosslinks (4) in their templates and mismatches at the 3'-end of the primer strand (5,6) has previously been studied.

We have used in vitro selection to isolate ribozymes that catalyze the ligation (7) and ATP(-γS)-dependent (thio)phosphorylation of RNA molecules (8). A number of these ribozymes yield modified RNA products: two types of ligases catalyze the formation of 2'-5' phosphodiester linkages and several classes of polynucleotide kinase ribozymes (thio)phosphorylate internal 2'-hydroxyls. Since the amplification step of the in vitro selection process requires reverse transcription, we were surprised to find such internally modified products. We expected that reverse transcriptase would have difficulty in reading through 2'-5' linkages; indeed, the ability of the enzyme to read through a template has been used as a criterion for the absence of such a linkage in an RNA (9). Since reverse transcriptase is known to stop at (or near) 2'-branch points in RNA (10-12), we also thought that the enzyme might have difficulty in reading through 2'-modified positions. In order to better understand the selective forces operating during in vitro evolution experiments and to explore the specificity of reverse transcriptase, we have characterized the behavior of two different reverse transcriptases, Avian myeloblastosis virus (AMV) and Maloney murine leukemia virus (MMLV), when they encounter 2'-5' linkages and 2'-thiophosphates in their template RNAs.

MATERIALS AND METHODS

Materials

AMV reverse transcriptase was purchased from Life Sciences (St Petersburg, FL) and Superscript MMLV RNase H + reverse transcriptase from Bethesda Research Labs. Ultrapure dNTPs and ddNTPs were from Pharmacia. All other salts, buffers and reagents used were of the highest possible grade (99%). The 2'-phosphitylated 3'-TBDMS 5'-DMT cytosine phosphoramidite was purchased from ChemGenes (Watertown, MA).

Preparation of substrates

The RNA substrate containing the 2'-5' linkage (Fig. 1a) was synthesized via solid phase phosphoramidite chemistry on an Expedite DNA synthesizer and deprotected overnight at 55°C in 3:1 aqueous NH₄OH:ethanol. The supernatant was dried under vacuum and treated with tetrabutylammonium fluoride at room temperature for 24 h. The fully deprotected RNA was purified by denaturing polyacrylamide gel electrophoresis. The class IV 2'-polynucleotide kinase ribozyme kin.10 (8) was synthesized by the T7 RNA polymerase run-off transcription of a PCR amplified DNA template. The 2'-thiophosphorylated RNA was prepared by allowing kin.10 RNA to react with 10 mM ATP-γS for 5 h as previously described (8). After gel-filtration to remove unreacted ATP-γS, the RNA was affinity purified by thiopyridine-activated thiopropyl sepharose (8). This RNA was >99.5% (based on affinity purification) modified with the 2'-thiophosphate.

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Reverse transcriptions

Primer extension reactions were carried out using the manufacturers recommended conditions. AMV RT reaction mixtures consisted of 50 mM Tris-Cl, pH 8.0, 60 mM KCl, 10 mM DTT, 6 mM MgCl₂, 375 μM each dNTP and 20 U AMV reverse transcriptase. The reaction mix for the MMLV H⁻ enzyme contained 50 mM Tris-Cl, pH 8.0, 40 mM KCl, 10 mM DTT, 6 mM MgCl₂, 0.5 mM each dNTP and 200 U Superscript MMLV H⁻ reverse transcriptase. Reaction volumes of 20 μl contained 1 μM RNA and ≤0.5 μM 5'-32P-labeled 3'-primer. Primer and template were heated to 70°C with all buffer components except MgCl₂ and enzyme, then allowed to cool to room temperature over 10 min, at which time the MgCl₂ was added. After equilibration to 42°C, reactions were initiated by the addition of enzyme. Aliquots were removed at various times, quenched by the addition of NaOH to 100 mM and heated to 90°C for 10 min to degrade the RNA template. Reaction mixes were neutralized with HCl, 2 vol of loading buffer (95% formamide, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, 0.05% xylene cyanol, 0.05% bromphenol blue) were added and aliquots were analyzed by denaturing polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

We examined the effect of a 2'-5' linkage and an internal 2'-thiophosphate in the template RNA on cDNA synthesis catalyzed by AMV and MMLV H⁻ reverse transcriptases.

2'-5' Linkage

A 36 nucleotide RNA (Fig. 1a) containing a single 2'-5' phosphodiester bond seven bases 5' of the primer binding site was synthesized by solid-phase phosphoramidite chemistry. This linkage, rather than completely blocking the progress of reverse transcriptase as expected, merely caused the enzyme to pause for several minutes. The pause occurred after having added the base opposite the 5' side of the 2'-5' phosphodiester (Fig. 2a). Under the conditions used for cDNA synthesis in the selection for RNA ligase ribozymes (7; 1 h incubation using MMLV H⁻ RT) this type of linkage is efficiently read through and there would have been little, if any, selective pressure against templates containing a 2'-5' linkage.

2' Thiophosphate

One of the class IV polynucleotide kinase ribozymes was allowed to auto-thiophosphorylate with ATP-γS to yield RNA containing a 2'-thiophosphate at a unique internal site. We had previously mapped the site of this modification, using partial alkaline hydrolysis, to the 2' hydroxyl of G62 (Fig. 1b) (8). The ribozyme is not labeled during incubation with β-32P-ATP-γS, demonstrating that thiophosphate is transferred rather than di- or tri-phosphate (Lorsch and Szostak, unpublished). This template modification also causes AMV and MMLV H⁻ reverse transcriptases to pause, but not to terminate reverse transcription (Fig. 2b). Both enzymes appear to pause predominantly before the 2'-thiophosphate, that is after adding the nucleotide across from C63 (Fig. 2b). There is also a minor pause of unknown origin opposite A64 and a pause of increasing relative intensity opposite G62: at early time points, the pause at C62 is stronger than the pause at G62 whereas at later time points the pause at G62 is stronger. This suggests that the enzyme reads to C62, pauses, slowly adds the C opposite G62 (the site of thiophosphorylation), pauses again and then finally continues on to make full length cDNA.

One possible alternative explanation for these data is that the ribozyme is not entirely specific for G62 and may thiophosphorylate the 2' hydroxyls of C63 and G61 to a limited extent. If there were three separate species, one 2'-thiophosphorylated at G61, one at G62 and one at C63, then one would expect the ratio of the band intensities corresponding to stops before each of these positions to remain constant (assuming that the sequence does not influence the severity of the pause). It is unlikely that some of the molecules are thiophosphorylated at G61 or C63 in addition to being thiophosphorylated at G62 because we do not see any aberrant products of nuclease P1 or T2 digestion as analyzed by DEAE cellulose thin layer chromatography (8).

When an RNA template with an 35S-labeled 2'-thiophosphate was reverse-transcribed for 60 min using the Superscript MMLV enzyme, the label was completely retained in the full-length translation products.
Figure 2. Primer extension of modified and unmodified RNAs using AMV and Superscript MMLV RNase H reverse transcriptases. Dideoxy sequencing lanes (not shown) were run next to the primer extension lanes. The sequences in the regions of the modifications are shown. (a) Primer extension on an RNA template containing a single 2'-5' linked phosphodiester bond and of a control RNA with only 3'-5' linkages. The samples were analyzed on a 15% denaturing polyacrylamide gel. (b) Primer extension on an unmodified RNA template and a template containing a single internal 2'-thiophosphate. The primer extension products were resolved on a 6% denaturing polyacrylamide gel.
double-stranded product, demonstrating that read-through is not due to the hydrolysis of the 2'-thiophosphate.

Under the conditions commonly employed for cDNA synthesis prior to each amplification step of an in vitro selection experiment, it appears that both 2'-5' phosphodiester and 2'-thiophosphate modifications are completely read through to give full length cDNAs. These results suggest that reverse transcriptases may be able to read through a variety of RNA backbone modifications. It is therefore difficult to predict whether or not ribozymes generating a given internal modification will be selected against during amplification of in vitro selected RNAs. It would be interesting to explore more fully the abilities of reverse transcriptases to read other modified and non-natural nucleic acids (e.g. completely 2'-5' linked RNA and DNA, PNA, etc.).

The above results show that reverse transcriptases pause at different positions depending on the modifications they encounter. The main pause for the 2'-thiophosphate modification is the base immediately before the phosphorylated nucleotide, whereas reverse transcriptases pause after reading through a 2'-5' linkage. Furthermore, 2'-thiophosphates also seem to produce two additional minor pauses, one nucleotide before and one nucleotide after the main pause. Similar effects have been seen with UV-induced cyclobutane dimer crosslinks in RNA (4). AMV reverse transcriptase stops after adding a base across from the position immediately 3' to the crosslink, but continues a certain fraction of the time to add a base across from the crosslinked nucleotide. In contrast, it was found that a crosslink produced by the aromatic azide SNAP [3-[(2-[(4-azido-2-nitrophenyl)amino]ethyl)dithio]propionate] resulted in a stop only at the base 3' to the site of modification. It is also known that AMV reverse transcriptase can read through abasic sites to give full-length cDNAs (1).

In light of these data, it would seem that reverse transcriptase primer extension mapping of the positions of crosslinks and other modifications should be interpreted cautiously since it is often unclear where the enzyme will stop (or pause) relative to a site of modification. Furthermore, the time of incubation can clearly affect the results. The fact that different enzymes have different fidelities and specificities (e.g. HIV RT; 2,6) suggests that the results of a primer extension experiment might also differ depending on the particular reverse transcriptase used.

REFERENCES