Tailing cDNAs with terminal deoxynucleotidyl transferase in RT–PCR assays to identify ribozyme cleavage products

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**ABSTRACT**

Polytailing a cDNA with terminal deoxynucleotidyl transferase (TdT) results in the addition of a homopolymeric sequence at its 3′-end. Here we describe the use of tailing in competitive RT–PCR assays to evaluate cleavage efficiency of ribozymes. Using a system that perfectly mimics intracellular cleavage, we were able to detect as few as 1% of cleaved moieties. Furthermore, employing primers overlapping the junction between tails and the cleaved RNA moiety in non-competitive assays, the sensitivity of the method could be improved to <10 fg. Using the latter protocol and reactions employing a trans-acting hairpin ribozyme targeting the nucleocapsid mRNA of the mumps virus, we were able to demonstrate ribozyme-induced cleavage.

Detecting ribozyme-induced trans-cleavage of RNA in cells is far from being a trivial issue. RNA-mediated catalysis in cells seems to be inefficient and reaction products can be quickly degraded by RNases (1). Common methods used to this purpose are northern blot and nuclease protection assays. Their optimization and application are technically fastidious and the detection threshold is, at best, 1 pg of RNA (2,3). Recently, Jones and Sullenger (4) achieved quantitative evaluation of intracellular cleavage for the group I ribozymes using a new method. The strategy is based on the specific property of this class of ribozymes to trans-splice a 3′-exon sequence onto substrate RNAs during cleavage. Unreacted substrate RNA and reaction products share the same sequence extremities but have different sizes and are amplified in competitive RT–PCR assays. Unfortunately, this approach cannot be applied to most of the therapeutically relevant ribozyme classes, like the ‘hammerhead’ and the ‘hairpin’ types.

We looked at the possibility of adapting the 5′-RACE (rapid amplification of cDNA ends; 5) procedures in an attempt to obtain such a method. 5′-RACE is a PCR-mediating methodology used to analyse mRNA 5′-ends when only a fragment of the sequence is known. The successive steps are: (i) cDNA synthesis using reverse transcriptase (RT) and a sequence-specific primer; (ii) poly-tailing of cDNA 3′-ends with TdT and a dNTP; (iii) PCR employing an homopolymeric primer and a second gene-specific primer; and (iv) cloning and analysis of PCR products.

We reasoned that if retrotranscription is initiated from a convenient reverse primer in the presence of the 3′-product of ribozyme cleavage, PCR with single-sided specificity of tailed cDNAs would result in competitive amplification of cleaved and uncleaved substrate moieties. An overview of the proposed protocol is presented in Figure 1A. Methods were applied and optimized in order to detect the 3′-product of a hairpin ribozyme (6) that cleaved the mumps virus nucleocapsid (NP) mRNA sequence in vitro at base 93 (GenBank accession no. X57997).

The method was investigated in a biological model system where known amounts of a cleaved-like RNA were introduced. This standard (bases 93–396 of the NP mRNA) mimics the 3′-cleavage product of the above hairpin ribozyme. To prepare it, DNA templates were generated by PCR (1) from the pMN2 plasmid (7) using a direct primer spanning the T7 promoter sequence followed by bases 93–110 of the mumps NP gene (5′-TATAAGCAGTCACTAC-TATAGGGTCGTCTGTGCTCAAAGC-3′) and a reverse primer corresponding to bases 379–396 of the same cDNA (5′-ACAGGGTGTTCTATCTGACG-3′). Finally, templates were purified (8) and transcribed using the T7 polymerase. Wild-type NP mRNA was obtained from Vero cells infected with the SBL-1 strain of the mumps virus. RNAs were harvested at 48 h post-infection, DNase treated, phenol extracted and quantitated by UV spectroscopy. RNA samples (5 µg) were then mixed with defined amounts of the artificial 3′-product of cleavage. Serial dilution of the standard ranged from 3.75 ng to 3.75 fg. Mixtures were reverse transcribed in reactions initiated from the above cited reverse primer, yielding cDNA fragments of 306 bases for the standard (including two extra guanosines that are added by the T7 polymerase; 9) and 396 bases for the native NP mRNA. cDNAs were purified from contaminating primers and nucleotides by three passages through Centricon 30 concentrators (Amicon) and polytailed with dCTP using the enzyme TdT. The tailed cDNAs were then amplified using a homopolymeric primer (dG9) and a nested NP primer (5′-GAGGGGAAGTGGAGAAATATGCTA-3′). Reactions were performed using the Expand PCR System (Boehringer Mannheim) and PCR products were resolved in 2% agarose gels.

PCR results showed competitive amplification of the standard mimicking the 3′-cleavage product in the range from 3.75 ng to 3.75 pg (Fig. 2A). A densitometric analysis of spots was performed and concentration at the titration point was calculated.

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Figure 1. Overview of procedures. (A) RNA substrate and the 3′-product of ribozyme cleavage are retrotranscribed, tailed and amplified by PCR using a homopolymeric primer (Pr H). (B) Specific amplification of the cleavage product is obtained employing a PCR primer composed of a homopolymeric stretch and a short specific sequence (PrH/S). Hatched boxes represent primers, small perpendicular lines indicate homopolymeric sequences and arrows indicate the direction of polymerase progression.

from plotted data (10). Assuming that this point is equivalent to 50% of cleavage, as little as 1% of 3′-products was detected.

To verify that the results do not reflect any preferential amplification of templates, solutions containing 5 μg of RNA from uninfected Vero cells and 200 pg of the RNA standard were mixed with a serial dilution of an RNA fragment (5′-NP) representing the native 5′ extremity of the NP mRNA (bases 1–396) and RT–PCR assays performed as above. The latter was prepared as described above, using a suitable direct primer (5′-TAA TACGACTCACTA TAGGGAAGCCAG-GAAGTGGTGTT-3′) in PCR amplification of DNA templates from the pMN2 plasmid. In the vicinity of the titration point the estimated values reflected the original amounts of RNAs added to the solutions (Fig. 2B). Thus, these experiments indicate that templates were amplified in a truly competitive way. They also showed that slipping of homopolymeric primers along tails during PCR cycling did not interfere with relative band density of PCR products.

The method was subsequently used to detect ribozyme-induced cleavage of the 5′-NP substrate. RNA from a Vero cell line producing the described hairpin ribozyme in a stable manner (J. Albuquerque-Silva et al., in preparation) was mixed with the substrate and reactions performed in MgCl₂-supplemented solutions as described elsewhere (6). At chosen times, samples were diluted (5′) and immediately retrotranscribed as described but using buffers without MgCl₂.

After amplification of the tailed cDNAs, no cleavage products were detected (data not shown). Considering that correct cleavage had occurred but to a low extent, we tried to improve the sensitivity of the method. This was done by the use of PCR primers spanning an homopolymeric stretch and overlapping into a short sequence complementary to the expected 5′-extremity of cDNAs in cleaved fragments (Fig. 1B). As primer specificity is largely dependent on correct annealing of its 3′-end, one can predict that such primers would be specific for cleaved fragments, thus avoiding competition during PCR with the over-represented wild-type RNA (Fig. 1B).

Preliminary studies showed that one sequence-specific base [primer: d(G)₉T] was enough to obtain the specific amplification of the truncated cDNA (Fig. 3A). Increasing the number of sequence-specific bases up to five, resulted in a concomitant increase in sensitivity. When primers with four to five specific bases [primer: d(G)₇GTCG or d(G)₇GTCGT] were employed, we were able to detect 7.5 fg of cleaved moieties (Fig. 3B). Primers with more than five complementary bases hybridize and amplify the cDNA of the native NP sequence (data not shown). Thus, five specific bases represents the upper limit for selective amplification of cleaved RNA. Primer d(G)₇GTCG was selected for further studies to avoid any risk of amplification of the
Figure 3. RT–PCR using primers spanning tailed cleavage junctions. The initial quantities of the RNA cleavage standard added to PCR reactions are indicated. cDNAs were polytailed with dCTP and PCR amplified as described in Figure 2. Primers d(G)8 GT (A) and d(G)7 GTCGT (B) were used for PCR amplification.

uncleaved NP mRNA. A further advantage in employing this primer was that, contrarily to primer d(G)7 GTCGT (Fig. 3B), no unspecific PCR products resulted from its use (Fig. 4).

The above cleavage reactions were repeated and detection of products proceeded as previously described, but using primer d(G)7 GTCG for PCR instead of the former homopolymeric primer. Results are shown in Figure 4. Cleavage products were detectable 1 or 3 h after starting reactions (lanes 3 and 4). Also, cleavage bands were absent in non-incubated reactions (t = 0, lane 2), suggesting that cleavage during retrotranscription, were it to exist, is undetectable. We concluded that the ribozymes produced in Vero cells are able to recognize and cleave their specific target sequence within the NP mRNA, but the reaction was largely inefficient as suggested by in vitro observations (6). Indeed, if >1% of the target was cleaved, the product should be apparent when the strictly homopolymeric primer is used in anchored RT–PCR assays.

Figure 4. Cleavage of the NP mRNA substrate by the hairpin ribozyme. Reactions were performed using RNA from Vero cells producing the hairpin ribozyme and the in vitro transcribed NP mRNA substrate containing the target sequence. Solutions of ribozyme-containing RNAs (5 µg) and in vitro transcribed NP mRNA substrate (230 pg) were separately adjusted to 15 mM in MgCl2, heated to 95°C for 1 min and allowed to cool. Reactions were initiated by adding the substrate to the enzyme-containing solutions at 37°C. At indicated times, samples were retrotranscribed, tailed and amplified using primer d(G)7 GTCG. The standard of cleavage (St) was used as positive control.

The described methods can be used to detect ribozyme-mediated cleavage of RNA and greatly simplify technical procedures. In addition, when compared with current protocols, the detection threshold increases by two orders of magnitude (<10 fg). Moreover, results suggest that when ribozymes target sequences are near the 5'-end of RNAs, quantitative estimation of the extent of cleavage can be obtained from competitive amplification of tailed cDNAs. This applies to the common cases of ribozymes targeting the 5'-untranslated region of mRNAs as well as to model substrates used to study RNA catalysis in cells.

REFERENCES