A simple method for 3′-labeling of RNA

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ABSTRACT

We describe a simple method for 3′-end labeling RNAs of known sequence. A short DNA template is designed to anneal to the 3′-end of the RNA, with a two nucleotide 5′ overhang of 3′-TA, 3′-TG or 3′-TC. The Klenow fragment of DNA polymerase I can then cleanly and efficiently extend the 3′-end of the RNA by the incorporation of a single α-32P-labeled dATP residue. This method can be used to label one RNA in a mixture of RNAs, or to label 5′-blocked RNAs such as mRNA.

Two methods are commonly used for 3′-end labeling RNA: T4 RNA ligase with 3′,5′-PpCp (1,2), and poly(A) polymerase with [α-32P]dATP (3). Labeling with T4 RNA ligase requires high concentrations of pCp and enzyme, and is less efficient with long RNAs (3). Poly(A) polymerase labels short RNAs poorly (3) and also requires a high concentration of [α-32P]CoTP for complete incorporation (4).

The natural role of DNA polymerases in the synthesis of Okazaki fragments by the elongation of RNA primers (6), and the demonstrated ability of DNA polymerase to extend an RNA primer on a DNA template (7) suggested a simple method for 3′-labeling of RNA. The ready availability of short synthetic oligodeoxynucleotides should allow any RNA of known sequence to be extended in a template-directed manner at its 3′-end, and therefore selectively labeled, by DNA polymerase in the presence of the appropriate dNTP (Fig. 1). After screening a number of polymerases, we found that DNA polymerase I large fragment [Klenow fragment (8,9)] is capable of rapidly and efficiently incorporating [α-32P]dATP onto RNA 3′-termini with minimal RNA degradation.

The labeling reaction is entirely template-dependent (Fig. 2). Templates containing 5′-OH or 5′-C, G or A overhangs did not lead to RNA labeling in the presence of dATP, whereas the template with a 5′-T overhang did lead to labeling. Templates with 5′-overhangs of TT, TTT, TTTT and TTTTTT were tested in order to determine the optimal length of the 5′ overhang. The oligo(T) templates led to much more efficient incorporation of dA. However the addition of increasing numbers of dA residues to the RNA resulted in a ladder of bands due to incomplete RNA extension (Fig. 2a). The degree of incorporation decreased only slightly from the bottom of the ladder to the top, but the incorporation of the last nucleotide was always much less efficient than the incorporation of the preceding nucleotides. The incorporation of [α-32P]dATP onto the RNA is ∼20-fold greater with a template overhang of 5′-T than it is with 5′-T. The same efficient incorporation is obtained using templates with an overhang of 5′-CT, AT or GT (Fig. 2b), which result in the termination of the extension reaction after the addition of only a single dA residue. These templates are superior for 3′-labeling because extension of the RNA is efficient and results in a single labeled product.

The time course of labeling was examined under the same conditions as used in Figure 2a. The extent of incorporation reached half maximal in ∼30 min and attained its maximum level in 2 h. The labeled product was stable and remained unchanged for several hours. After 24 h, some loss of label from the RNA was observed, possibly as a result of the 3′–5′ exonuclease activity of the Klenow fragment enzyme. In the presence of high concentrations of DNA template, this degradation by the enzyme is largely prevented.

The high affinity of DNA polymerase for polynucleotides (Km = 5 nM, DNA) (10) and for dNTPs (Km = 1–2 µM) (11) leads to the rapid labeling of low concentrations of RNA, or, if higher RNA concentrations are present, to the incorporation of a large fraction of the labeled dNTP into RNA product. The extent of incorporation of [α-32P]dATP in a 2 h incubation is dependent on the concentration of RNA, over the range from 0.5 to 500 nM. Higher RNA concentrations lead to incorporation of a higher fraction of the [α-32P]dATP (up to 90%, data not shown), and conversely, increasing the dATP concentration to 10–100 µM led to labeling of a greater fraction of the RNA molecules (Fig. 2c). In the presence of 10 µM dATP, 100 nM RNA 40 was nearly quantitatively converted by Klenow into the single base addition product.

The effect of varying the template DNA concentration was determined for 12 and 17 nt templates with 10- and 15-nucleotide RNA/template overlaps, respectively (Fig. 2d). RNA labeling was maximal in the presence of 100 µM of the 12mer DNA template, with 10µM template yielding 20% of maximal labeling efficiency. In contrast, 100 nM of the 17mer template was saturating for the extension of 50 nM RNA. We also observed template-directed 3′-labeling of DNA under the same conditions. The efficiency of labeling of DNA 40 using the 12mer template was about seven times less than that of RNA 40, probably because of weaker binding of the DNA template to a DNA primer as opposed to an RNA primer. Longer templates should therefore be used when labeling DNA fragments.

Since the 3′-labeling of RNA is a template-dependent polymerization, one RNA in a mixture of RNAs can be selectively

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labeled. In a mixture of RNA 40 and RNA 50 (see Fig. 2 for sequences), RNA 40 was specifically labeled in the presence of its complementary template, and RNA 50 was labeled when its template was added (data not shown). Both RNA 40 and RNA 50 were labeled when both templates were added. This principle could be applied to selectively label one RNA species in a complex mixture could also be useful (14); for example, T7 RNA polymerase often generates RNAs with heterogeneous 3′-termini—it should be possible to label just one of these transcripts. For some experiments it may be desirable to label an RNA with a terminal ribonucleotide, dideoxy-ribonucleotide or other modified nucleotide. Unfortunately, labeling with [\alpha-\text{32P}]dATP or [\alpha-\text{32P}]dTTP was very inefficient (in buffer without Mn2+), probably because of the low affinity of the DNA polymerase for ribonucleotide and dideoxy-ribonucleotide triphosphates. However, recently described mutants of DNA polymerase with relaxed specificity may allow the efficient incorporation of a terminal ribonucleotide onto an RNA primer (C. M. Joyce, personal communication).

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