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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-5′, 3′-TG-5′ or 3′-TC-5′. 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-^{32}P-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′[5′-^{32}P]pCp (1,2), and poly(A) polymerase with [α-^{32}P]cordycepin 5′-triphosphate (CoTP or 3′-deoxy-ATP) (3,4). 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-^{32}P]CoTP for complete incorporation (5).

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-^{32}P]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 TTTTT 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-^{32}P]dATP onto the RNA is ~20-fold greater with a template overhang of 5′-TT 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 (K_m = 5 nM, DNA) (10) and for dNTPs (K_m = 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-^{32}P]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-^{32}P]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 10 μM 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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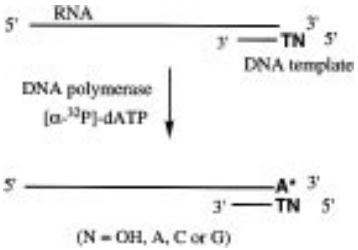

Figure 1. Schematic representation of 3′-terminal extension of an RNA primer on a DNA template.

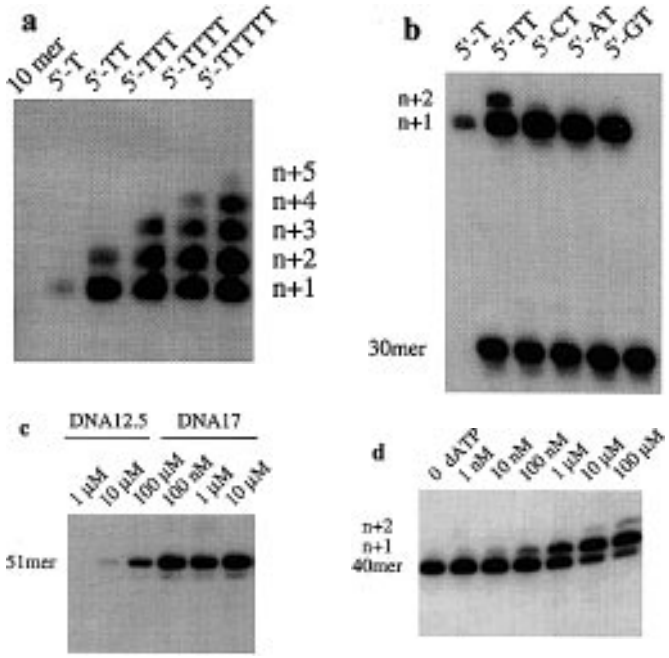

Figure 2. Effect of different templates and of varying template and dATP concentrations on 3′ labeling reaction. (**a**) Templates were synthetic oligodeoxynucleotides with the sequence 5′-GGGTTGCTGG-3′ preceded by 0–5 Ts (100 μM in each reaction). Each labeling reaction mixture also contained RNA 40 (5′-GGGTTGGGAAGAAACTGTGGCACTTCGGTGCCAGCAACCC-3′, 5 nM), cold dATP (2 μM) and [α-^{32}P]dATP [(3000 Ci/mmol, 10 mCi/ml), 66 nM]. (**b**) Templates and reaction conditions were as above, with 5′ overhangs as indicated. Templates beginning 5′-CT, 5′-AT and 5′-GT allow efficient incorporation of a single dA residue. (**c**) The concentration of templates DNA 12.5 (5′-GTGTAGTTGAAT-3′) and 17 (5′-GTGTAGTTGAATCAGCA-3′) was varied from 100 nM to 100 μM. Reaction mixtures contained RNA 50 (5′-GGAGAGTATGCAGTAGTCATCGCGACGTAGCTAGATGCTGATTCAACTAC-3′, 100 nM) and [α-^{32}P]dATP [(3000 Ci/mmol, 10 mCi/ml), 66 nM]. (**d**) The concentration of unlabeled dATP was varied from 0 nM to 100 μM, and the conversion of RNA to the 1 nt longer 3′ extension product was followed. Reaction mixtures contained template DNA 12.4 (5′-GTGGGTTGCTGG-3′, 100 μM) and 5′ ^{32}P-labeled RNA 40 (100 nM).

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 also be used to label a specific DNA of known sequence in a mixture of different DNAs.

We have successfully labeled mRNA from human leukemia T cells by 3′-primer extension. Klenow fragment and [α-^{32}P]dATP alone will not label mRNA, however, in the presence of $(dT)_{20}$, which binds to the 3′-poly(A) tail, efficient 3′-labeling of the mRNA was observed (data not shown).

We have described a simple procedure by which the 3′-termini of RNA molecules can be labeled by using the Klenow fragment of *E.coli* DNA polymerase I to extend the 3′-end of the RNA by one nucleotide on a short complementary DNA template. This method requires that the sequence of the 3′-end of the RNA be known, and that a complementary template be synthesized. However, the labeling process itself is rapid and efficient, and yields a single homogeneous labeled product. The key to increasing the efficiency of labeling to a practical level is the use of a template with a 5′ overhang of at least two nucleotides, but with a sequence (e.g. 5′-AT, 5′-CT or 5′-GT) that will cause the extension reaction to terminate after the incorporation of only one labeled nucleotide (dA in these examples).

The 3′-labeling procedure described above should be useful for a variety of purposes, such as following a 3′ RNA fragment in ribozyme or RNA processing reactions, and the labeling of 5′ blocked RNAs such as mRNAs (12,13). The ability 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, dideoxynucleotide or other modified nucleotide. Unfortunately, labeling with [α-^{32}P]ATP or [α-^{32}P]2′,3′-ddATP was very inefficient (in buffer without Mn^{2+}), 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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