METHODS REPORT

Tailing and 3′-end labeling of RNA with yeast poly(A) polymerase and various nucleotides

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ABSTRACT

We have tested conditions for the labeling and tailing the 3′-end of RNAs with yeast poly(A) polymerase. Conditions were optimized for addition of NTP, dNTP, or ddNTP nucleotides to RNA. ATP, GTP, and UTP were useful for adding homopolymer tracts of various lengths. The nonradioactive nucleotides biotin-N^6-ATP and digoxigenin-11-UTP also were used efficiently.

Keywords: biotin-N^6-ATP; digoxigenin-11-UTP; digoxigenin-16-dATP; fluorescein-12-ATP; monoaddition

INTRODUCTION

For 3′-end labeling of RNA, several methods have been described involving radioactive reagents: with T4 RNA ligase, [5′-^{32}P]pCp is added (England & Uhlenbeck, 1978), and with bacterial and yeast poly(A) polymerase (PAP), [α-^{32}P]-cordycepin 5′-triphosphate (CoTP or 3′-dATP) is transferred to the RNA 3′-ends (Winter & Brownlee, 1978; Lingner & Keller, 1993). If the sequence of the RNA is known, 3′-end labeling can be performed with a DNA oligo and Klenow polymerase (Huang & Szostak, 1996).

Nonradioactive methods for RNA labeling mainly involve in vitro transcription with T3-, T7-, or SP6-RNA polymerase and digoxigenin-11-UTP. The enzyme terminal nucleotidyl transferase (TdT) is also able to add digoxigenin-11-dUTP to RNA molecules (Rosemeyer et al., 1995).

Besides 3′-end labeling, other uses for yeast PAP have been reported, such as the polyadenylation and subsequent purification of *Escherichia coli* messenger RNA (Amara & Satchidanandam, 1997), or the determination of RNA extreme 3′-end sequences (Lingner et al., 1994; Zaug et al., 1996). Yeast PAP was also found to be suitable for 3′-end labeling of an RNA dinucleotide containing a 3′-S-phosphorothiolate linkage (Weinstein et al., 1996).

To explore further applications for recombinant yeast poly(A) polymerase in RNA 3′-end labeling, we tested most of the available ribo-, deoxy-, and dideoxynucleotides as substrates. In addition, we also determined the efficiency of incorporation of four nonradioactive nucleotides.

RESULTS AND DISCUSSION

3′-End labeling with different types of nucleotides

In a first attempt, we tested the addition of rNTPs, dNTPs, and ddNTPs to a 15-mer oligo(A) RNA primer [$(A)_{15}$]. Under nonspecific conditions (Wahle, 1991), nucleotides were transferred to the primer to various degrees (Fig. 1A). With 500 ng of PAP, between one and four residues of the deoxynucleotides were added to $(A)_{15}$, previously labeled at the 5′-end with [γ-^{32}P]ATP. Of the ribo-NTPs, GTP and UTP were incorporated more efficiently than CTP. Striking was the elongation of $(A)_{15}$ with a 14-nt GMP-tail, suggesting an application for G-tailing of a defined length (see below). In these experiments, the nucleotide concentration was 0.5 mM, which explains the addition of more than one nucleotide, particularly of dATP and UTP. Most of the chain-terminating dideoxynucleotides were transferred to $(A)_{15}$ with efficiencies between 40 and 80% (Fig. 1A). As expected, the reaction with CoTP went to completion (Lingner & Keller, 1993).
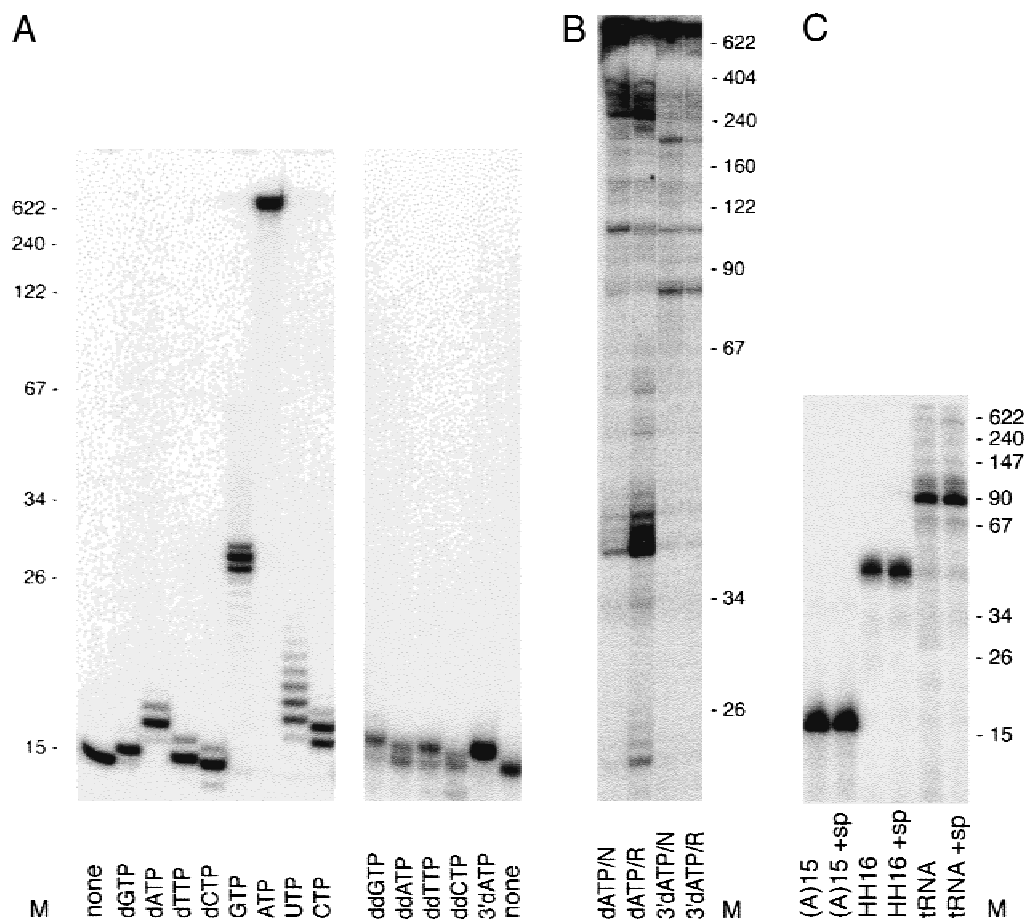
FIGURE 1. A: Elongation of $(A)_{15}$ primer with various nucleotides. $(A)_{15}$ (5′-labeled with polynucleotide kinase and [γ-^{32}P]ATP; 3.8 pmol) were incubated in YUN-buffer with 9 pmol of PAP and 0.5 mM nucleotides in 10 μL reaction volume for 30 min at 37 °C. After addition of 6 μL of formamide/bromophenol blue loading buffer, 4 μL were separated on a 16% sequencing gel. Gels were exposed to a PhosphorImager screen and analyzed with the IP-labgel software (Molecular Dynamics). **B:** Labeling of calf thymus total RNA with [α-^{32}P]dATP or [α-^{32}P]-CoTP. Total calf thymus RNA (143 ng) was incubated with 250 ng of PAP for 30 min at 37 °C in YUN-buffer and separated on a 16% polyacrylamide gel. Reactions (5 μL) contained 0.25 μCi (3,000 Ci/mmol) of [α-^{32}P]dATP (lanes 1 and 2) or [α-^{32}P]CoTP (lanes 3 and 4). Reactions in lanes 2 and 4 were run with a temperature gradient from 25 to 60 °C within 30 min. **C:** Labeling of the RNA primers $(A)_{15}$, hammerhead-16 ribozyme, and tRNA and effect of spermidine. Primer (20 pmol) was incubated in YUN-buffer with 500 ng of yeast PAP. Reactions (20 μL) also contained 4 pmol of dATP and 0.5 μCi of [α-^{32}P]dATP. Reactions run in (+sp) lanes contained 1 mM spermidine. The reaction was incubated for 30 min at 37 °C. Loading buffer (14 μL) was added and 5 μL were run on a 12% polyacrylamide gel.

In a further experiment, we tested the use of [α-^{32}P]dATP for the end labeling of total RNA from calf thymus (Fig. 1B). In order to label most of the RNA molecules by addition of a single nucleotide, a molar ratio of about 1:10 of NTP:RNA was used. A temperature gradient from 25 to 60 °C in 30 min was found to increase the efficiency of labeling of certain RNA species, presumably because of RNA unfolding at elevated temperatures. Also, some RNAs are labeled more strongly with dATP than with CoTP and vice versa. Labeling with dATP might therefore be an alternative to CoTP because of a different labeling pattern. [α-^{32}P]dATP has been used to end-label total RNA from yeast, in order to measure poly(A) tail lengths (Kessler et al., 1997).

Furthermore, ATP can be used easily for labeling of the 3′-end of RNAs, in particular, when a free terminal 3′-OH is desired. Because nucleotidyl transfer of PAP is distributive, the use of equimolar amounts of ATP to RNA in the reaction leads to a Gaussian distribution of oligoadenylation. We tried to find conditions for monoaddition of ribonucleotides by substituting MgCl$_2$ for MnCl$_2$ or by decreasing the PAP concentration in the reaction, but always observed an inverse correlation between efficiency of transfer and monoaddition. Therefore, if labeled RNA primers of a defined length are required, AMP-labeled RNA molecules must be fractionated on a polyacrylamide gel (Frendewey & Keller, 1985).
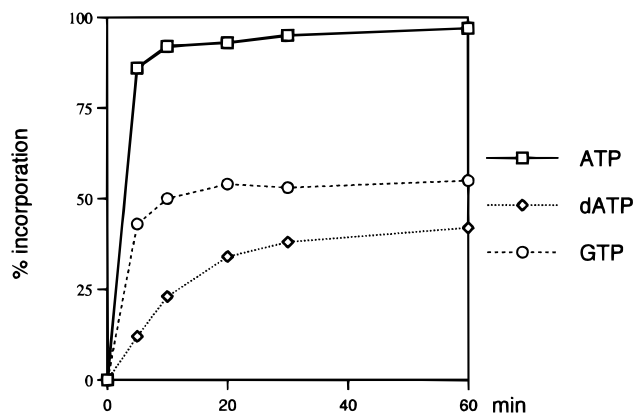
We tried several means to improve the efficiency of labeling RNA with PAP. Figure 1C shows that the use of spermidine stimulates the labeling of $(A)_{15}$, hammerhead-16 ribozyme RNA HH16 (Hertel et al., 1996), and tRNA with [α-^{32}P]dATP approximately twofold, and this was more pronounced with longer RNAs.

Labeling of some RNA species was also improved by applying a gradient of increasing temperature during the reaction (Fig. 1B). Figure 1C shows that, with dATP in sub- or equimolar amounts relative to the primer, most products are extended by one nucleotide only.
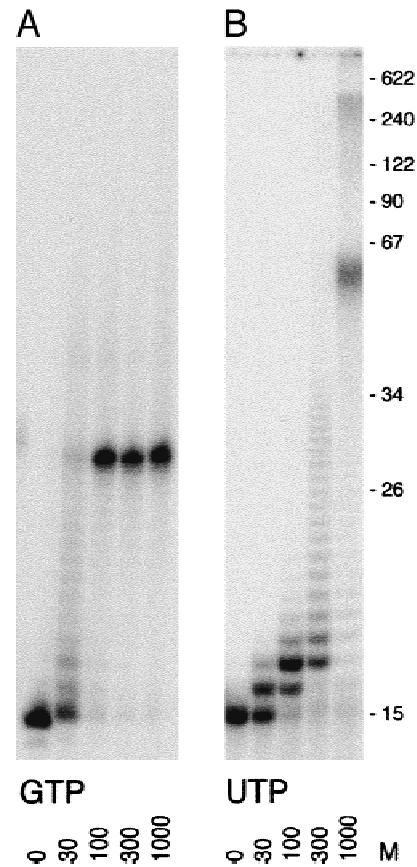
When we tested the three substrates [α-^{32}P]ATP, [α-^{32}P]dATP, and [α-^{32}P]GTP under conditions where the RNA was in excess over label, we found that [α-^{32}P]ATP was incorporated to almost 100% (Fig. 2). [α-^{32}P]dATP and [α-^{32}P]GTP were incorporated somewhat less efficiently (55% and 35%, respectively). We emphasize that the reaction conditions should be optimized for different RNA species. In this context, we found that the specific activity of PAP varies dramatically with the length of the RNA substrate and with the complexity of its expected folding. PAP has no activity on RNA with a hairpin structure and blunt ends (unpubl.). It is most active on a homooligomer like (A)$_{15}$ as used in these experiments (2.5×10^7 units/mg). Poly(A) contains a much lower number of intact 3'-ends (Wahle, 1991) and PAP has an activity of $1.2–2.4 \times 10^6$ units/mg with this substrate. On highly folded RNA molecules, such as tRNA and hammerhead-16 ribozyme, the activity is intermediate.

Tailing of RNA with ATP, GTP, and UTP

Although ATP is the most obvious choice for tailing of RNA, GTP and UTP were also found to be useful. The addition of GMP-homopolymer tracts to RNA by Vaccinia PAP has been reported previously (Thomson & Gershon, 1995). About 14 GMP residues can be added to an RNA primer in this way. We could reproduce this (G)$_{14}$-tailing reaction with yeast PAP (Fig. 3A) and with

FIGURE 3. A: GTP-tailing. Polynucleotide kinase-labeled (A)$_{15}$ (20 pmol) was incubated in 10-μL reaction mix containing YUN-buffer and 0.5 mM GTP for 30 min at 37 °C. The amount of PAP in the reaction was varied between 30 and 1,000 ng, as indicated. **B:** UTP-tailing. The reaction conditions were as for A, except that UTP was used as substrate.

recombinant bovine PAP (Wahle et al., 1991) (not shown). In a mobility shift experiment, we found that yeast PAP binds very inefficiently to a primer containing a (G)$_{14}$ tail (unpubl.). The low affinity of enzyme binding might therefore explain this phenomenon. This method can be useful for applications where the addition of a short G-tail is desired.

With UTP as substrate, PAP preferentially adds a track of about 40 UMP nucleotides to RNA primers (Fig. 3B). However, clusters of other lengths were sometimes observed in addition to the main product.

Labeling of RNA with nonradioactive nucleotides

Nonradioactive labeling of hybridization probes is widely used in Southern and northern analysis and DNA library screens (Schnorf et al., 1991), as well as in in situ hybridization experiments on chromosome squashes (Langer-Safer et al., 1982) and tissue sections (Tautz & Pfeifle, 1989; Hauptmann & Gerster, 1996). Further applications are DNA sequencing with fluorescent markers and analysis of DNA, RNA, or pro-

FIGURE 2. Efficiencies of incorporation of [α-^{32}P]-labeled nucleotides. The relative amount of incorporated nucleotides [α-^{32}P]ATP, [α-^{32}P]GTP, and [α-^{32}P]dATP were measured. The adenylation reaction was done in 25 μL of YUN-buffer. Nucleotides (6.6 pmol), (A)$_{15}$ (20 pmol), and yeast PAP (500 ng) were present in the reaction. One-microliter aliquots were spotted onto DE-81 filters at the times indicated. The filters were then washed and counted in a scintillation counter as described (Martin & Keller, 1996).

teins containing fluorescent markers on special work-stations. We tested the ability of yeast PAP to label RNA with biotin-N^6-ATP (BIO-ATP), fluorescein-12-ATP (FLUORO-ATP), digoxigenin-16-dATP (DIG-dATP), and digoxigenin-11-UTP (DIG-UTP). Of the four nucleotides, BIO-ATP was the most efficient substrate for nonradio-active labeling (Fig. 4). The detection limit of the labeled RNA in a spot test with streptavidin-conjugated alkaline phosphatase was 0.1 fmol. Surprisingly, when BIO-ATP was used alone, almost no incorporation was observed. If regular ATP was added to the reaction in amounts of one- or twofold that of BIO-ATP, the reaction was strongly stimulated. The other relatively efficient substrate was DIG-UTP, with a detection limit of 10 fmol. With FLUORO-ATP, the detection limit was around 100 fmol and DIG-dATP had a rather low efficiency of incorpo-ration and only 1 pmol was detectable. Our results with BIO-ATP are therefore superior to results with terminal transferase. This enzyme is able to tail RNA at the 3′-end with digoxigenin-dUTP (Rosemeyer et al., 1995). A detection limit of 1.2 fmol was reported with a 25-mer RNA oligo as primer and longer RNAs were rather in-efficiently labeled. With yeast PAP, the yield in labeling tRNA was only slightly lower than for $(A)_{15}$ (see also

FIGURE 4. Efficiency of labeling RNA with yeast PAP and nonradio-active nucleotides. $(A)_{15}$ (20 pmol) was incubated at 37 °C for 40 min with 500 ng of yeast PAP in YUN-buffer and varying concentrations of BIO-ATP, DIG-dATP, DIG-UTP, and FLUORO-ATP as indicated. To the left, the ratio of labeled:unlabeled nucleotide in the reaction is indicated, where 1 equals 0.1 mM, except for DIG-dATP, where 1 equals 0.05 mM. Numbers below the filter indicate amounts of RNA spotted in volumes of 1 μL.

Fig. 1C). With an in vitro-transcribed RNA of 700 nt length, we found the labeling efficiency with BIO-ATP to be several hundred-fold lower than with $(A)_{15}$. This may also depend on the folding of the RNA and whether the 3′-end of the primer is readily accessible.

It is well possible that several of the commercially available nucleotides with nonradioactive markers are also efficient substrates for PAP. Unfortunately, DIG-ATP is currently not available commercially. Another application for RNA labeled with BIO-ATP could be the streptavidin-coated paramagnetic bead technology (Shepard & Rae, 1997) for the isolation of rare cDNAs.
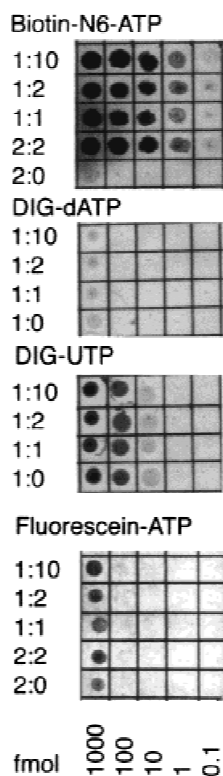
MATERIALS AND METHODS

Materials

Recombinant yeast PAP used in all experiments was pre-pared as described (Lingner et al., 1991a) and corresponds to the enzyme commercially available from Amersham. The specific activity is 2.5×10^7 units/mg with $(A)_{15}$ as primer (Lingner et al., 1991b). tRNA and nucleoside triphosphates were from Boehringer-Mannheim except BIO-ATP and FLUORO-ATP were from NEN. $[\alpha\text{-}^{32}P]ATP$, $[\gamma\text{-}^{32}P]ATP$, $[\alpha\text{-}^{32}P]dATP$, $[\alpha\text{-}^{32}P]GTP$, and $[\alpha\text{-}^{32}P]3'\text{-}dATP$, all at 3,000 Ci/mmol, were from Amersham. The hammerhead-16 ribozyme template was a gift from Neena Grover and Olke Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado, Boulder) and had the sequence: GGGAACGTTT CGGCCTTTCG GCCTCATCAG GTCATCGCTA TAGTGAG TCG TATTA.

Adenylation reactions with yeast PAP

Standard reactions were done in yeast PAP nonspecific as-say buffer (YUN-buffer), which consisted of 10% glycerol, 25 mM Tris-HCl, pH 7.0, 0.1 mg/mL BSA, 0.7 mM $MnCl_2$, 50 mM KCl, 0.01 mM EDTA, and 0.5 mM DTT.

Detection of biotin-, digoxigenin-, and fluorescein-nucleotide labeled RNAs

All the reagents and the nylon membrane for the detection were from Boehringer-Mannheim. The labeling reactions were stopped with an equal volume of RNA dilution buffer ($6\times$ SSC, 5 mM EDTA, 0.1% SDS, and 50 μg/mL of tRNA). Serial 1:10 dilutions were done in RNA dilution buffer. One-microliter aliquots were spotted onto nylon membrane and crosslinked to the filter with a UV device (Stratalinker-1800) in the au-tocrosslink mode. Filters were processed according to the DIG-System manual from Boehringer-Mannheim. Filters con-taining BIO-ATP were probed with Streptavidin-alkaline phos-phatase conjugate, diluted 1:5,000 in blocking solution. Filters with fluorescein-ATP assays were incubated with anti-fluorescein-alkaline phosphatase, Fab fragments, diluted 1:5,000 in 1% blocking solution, and filters containing DIG-UTP and DIG-dATP were probed with anti-digoxigenin-alkaline phosphatase, Fab fragments at the same dilution. The alka-line phosphatase color reaction was done using NTB/BCIP tablets.

ACKNOWLEDGMENTS

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